

# THE MOVING BOUNDARY METHOD OF STUDYING THE ELECTROPHORESIS OF PROTEINS

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*INAUGURAL DISSERTATION*

BY

**ARNE TISELIUS**

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BY DUE PERMISSION OF THE PHILOSOPHICAL  
FACULTY OF UPSALA TO BE PUBLICLY DIS-  
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## Introduction.

### The first electrophoresis experiments.

In 1807 a Russian physicist REUSS (1) made an experiment that must be regarded as the first observation of electrophoresis and electroosmosis. He placed two vertical glass tubes in a piece of wet clay and filled the tubes with water. On the bottom of the tubes was a layer of fine sand. The wires from the poles of a Voltaic pile with 74 couples of silver rubles and zinc pieces were led into the tubes.

When the current was closed, he observed that at the positive pole the water became milky by the migration of fine clay particles through the sand layer. At the negative pole the water remained clear, but increased in volume.

After REUSS' observations of the migration of suspended particles, a great number of investigations of the phenomenon were made, among others by FARADAY and by DU BOIS-REYMOND (2). Still its nature was very doubtful until quantitative investigations were made by WIEDEMANN (3) and by QUINCKE (4). From QUINCKE's measurements it became evident that the rate of migration of suspended particles is proportional to the potential gradient, even if he did not himself clearly point out this fact.

PICTON and LINDER (5) in 1892 published a paper entitled »A new Property of certain Solutions» in which they describe some experiments concerning the action of an electric current upon colloidal solutions. They immersed electrodes connected with the poles of a storage battery in a colloidal solution of arsenious sulphide and observed that the sulphide gradually sank below the electrodes. The experiment was repeated in a V-shaped tube, where it was found that the sulphide descended from the negative electrode, leaving colourless liquid behind. Other colloids, for instance shellac, ferric hydrate, hemoglobin, and magdala red showed similar behaviour. To prove that the migration was not due to any irreversible changes, they reversed the direction of the current, and then observed migration in the opposite direction.

With regard to the explanation of this new phenomenon PICTON and LINDER said: »It may be that the aggregates in these solutions are in an electrified condition, but on this subject it would be rash to dogmatise at present». However, they pointed out the analogy to the electric migration of acidified water through membranes (electroosmosis).



In 1897 they published a report on some further experiments (6). It was found that various colloids in a vertical tube migrated downwards with a sharp boundary when an electric current was sent through. For some substances this occurred when the upper electrode was positive, for others when it was negative. They stated that the direction of migration seemed to be in relation to the acidic or basic character of the colloid.

Among the now rapidly increasing number of observations on the migration of colloids the experiments of W. B. HARDY (7) are of special interest for this work since they deal with proteins, and are of importance for the development of quantitative methods.

HARDY worked first with an apparatus similar to that of LINDER and PICTON, with the electrodes placed directly into the colloid, but later he used a U-tube apparatus where the colloid (an opalescent globulin solution) was allowed to form a layer under a solution containing the same amount of electrolyte. With this apparatus he was able to make quantitative determinations by observing the rate of migration of the boundaries. He found that the mobility obtained was of the same order of magnitude as that of ordinary ions ( $20 \times 10^{-5}$  cm./sec. per volt/cm. at  $13^\circ$ ).

The method of measurement in this work of HARDY was similar to that earlier used by several workers for the measurement of ionic mobilities.

I will not go into further detail about the development of the study of electrophoresis in general (8); it may be sufficient to remark that work since the first investigations mentioned above has been mainly devoted to the study of the influence of electrolytes on mobility, on account of the relation that seems to exist between this effect and coagulation.

### The development of the moving boundary method.

The problem of measuring electrophoretic migration velocities of particles not visible in the ultramicroscope is in principle in no way different from the problem of measuring ionic velocities, and the same two methods, the moving boundary method and the transference method, can be used for both purposes. It must be remembered, of course, that the special properties of colloids may make special precautions necessary. It is, however, remarkable how slowly the experiences made with ordinary electrolytes were applied in electrophoresis measurements. This is especially true of the moving boundary method.

This method was first used for measuring ionic migration velocities by OLIVER LODGE (9) and was worked out further by WHETHAM (10), NERNST (11), MASSON (12), and DENISON and STEELE (13). Lately the method has been used by McINNES and collaborators (14).

WHETHAM studied the migration of the boundary between two salts  $AC$  and



$BC$  with a common ion  $C$ . He tried to choose for  $AC$  and  $BC$  two salts of as nearly the same specific conductivity as possible. In fact, it is evident that only if  $A$  and  $B$  possess the same mobility, the boundary will migrate through the tube with the same velocity as the ions  $A$  and  $B$  and no other changes take place. The question what will happen when  $A$  and  $B$  have different mobilities was also discussed by WHETHAM and later, in more detail, both experimentally and theoretically, by MASSON. WHETHAM found that if  $A$  migrates in front of  $B$  and the mobility of  $A$  is greater than that of  $B$  the boundary  $A/B$  will remain sharp. In other cases the boundary is not stable and mixing will take place. MASSON deduced that this sharp boundary must migrate with the velocity of the ion  $A$  in the potential drop existing in the solution  $AC$ . Since the ions  $B$  have a smaller mobility, the potential gradient below the boundary must be higher in order that the ions  $B$  may be able to migrate with the velocity of the boundary. Therefore the concentration of  $BC$  below the boundary must have a value  $c_{BC}$  corresponding to a conductivity  $\kappa_{BC}$  so that

$$u_B/\kappa_{BC} = u_A/\kappa_{AC} \quad (1)$$

where  $u_A$  and  $u_B$  are the mobilities of the ions  $A$  and  $B$ .

In this case, and only in this case, the boundary can remain sharp. If  $c_{BC}$  from the beginning has a higher value, we still get a sharp boundary migrating with the same velocity, and followed by a column of  $BC$  of the right concentration, only in this case another boundary, that does not move, appears at the place of the original boundary. Here the concentration of  $BC$  changes from the original to the right value (fig. 1).

It should be observed that the moving boundary remains very sharp during the experiment. If an ion  $B$  were to diffuse into the region of the ions  $A$ , it would come into a region of lower potential gradient, where its velocity is smaller and it would soon be left behind the  $A$ -ions and reached by the boundary again.

Some years before MASSON's paper appeared, KOHLRAUSCH (15) and WEBER (16) published papers containing a more general theory of the migration of discontinuities in electrolyte solutions. In a following chapter their equations will be applied to some cases of importance for this work (p. 17). Here I will only mention that they arrived at the results related above for the case  $AC/BC$ . For cases not quite as simple the phenomena become very complicated, especially if the variation of the mobilities with concentration has to be taken into account.

In all determinations of ionic mobilities by the moving boundary method, the simple case  $AC/BC$ , considered here, has been studied.  $A$  is the ion, the mobility of which is to be determined,  $B$  is »the indicator» chosen so that its mobility is lower than to be expected for  $A$ , and the velocity of the boundary gives the mobility of  $A$ . The results in most cases seem to agree well with those obtained by the

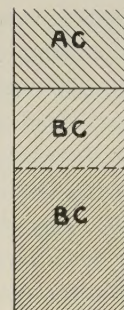


Fig. 1.



transference method. Still there seems to be some doubt about the general applicability of this method. Some authors report different results with different »indicators» (17). The automatic adjustment of the concentration claimed by KOHLRAUSCH and WEBER only seems to take place in a rather narrow range of concentration (14). The influence of diffusion is still very obscure; KOHLRAUSCH and WEBER have not taken into account the diffusion in their calculations and for the treatment of the migration of the boundaries they must assume that these remain completely sharp.

The objections against the moving boundary method are of course still more justified in its application to the measurement of the electrophoresis of colloids. In a colloid small quantities of electrolytes are always present and usually we do not know exactly which substances are present and in what concentrations. For such mixtures we cannot apply the procedure described above, where it was assumed that on each side of the boundary only one electrolyte was present. In fact no measurements of electrophoresis have been made in this way. Most workers have simply made use of a salt solution, such as KCl, as a supernatant liquid and chosen its concentration so that the conductivity is equal to that of the sol, in order to obtain a constant potential gradient throughout the range of migration. It is evident that this method is very doubtful. The salt solution very soon diffuses down into the sol layer and causes quite uncontrollable changes in mobility and potential gradient. Compared to ions of ordinary electrolytes it is, indeed, a characteristic of colloids that as regards mobility the former are very much more sensitive towards small changes in the ionic medium than the latter. Moreover, according to the facts mentioned above, the supernatant ions may directly influence the movement of the boundary.

That complications appear in electrophoresis measurements performed in this or similar ways has probably been evident to most workers. For example, one very often finds the statement, that it was only possible to make measurements on one boundary; the other was completely blurred. Already HARDY, in his work of 1905 (7), emphasized very clearly the necessity of taking the above mentioned effects into account in work with colloids. His measurements, as described above, were performed under conditions that must still be considered to be the only ones permissible for colloids: equal ionic medium on both sides of the boundary. He also made a special investigation (with methylene blue) of the quantity salt necessary to minimize the differences in medium on both sides of the boundary sufficiently to give the same rate of migration of the two boundaries in the U-tube. His results form a good example of the importance of these factors and therefore a table from this work is given below. It gives the migration (in mm. in 10 min.) of the boundaries in a solution containing 1 mol of methylene blue in 144.93 litres and varying amounts of magnesium and potassium sulphate.



Table I.

|                                                      | —  | +     | mean |
|------------------------------------------------------|----|-------|------|
| 0.0476 n $\text{MgSO}_4$ in both layers . . . . .    | 4  | ↑ 4 ↓ | 4    |
| 0.0244 n   "   "   "   "   " . . . . .               | 4  | 4     | 4    |
| 0.0012 n   "   "   "   "   " . . . . .               | 6  | 2     | 4    |
| 0.0000 n . . . . .                                   | 22 | 1     | 11.5 |
| 0.0476 n $\text{K}_2\text{SO}_4$ "   "   " . . . . . | 4  | 4     | 4    |
| 0.002 n   "   in upper layer only . . . . .          | 20 | 3     | 11.5 |
| 0.009 n   "   "   "   "   " . . . . .                | 4  | 2     | 3    |

Later, DUCLAUX (18) also pointed out the difficulties connected with these effects (for which the name »boundary anomalies» will be used in the following). In his work he used the transference method instead.

On account of its convenience the moving boundary method has been widely used, even if an increasing number of authors criticized its application (8, 17, 19, 20).

However, some attempts have been made to overcome the difficulties, but without any great success. MUKHERJEE (21) describes an apparatus where the potential gradient in the neighbourhood of the boundary is measured with two additional electrodes. Even if in this way we really succeeded in measuring the potential gradient at the very boundary, there still remains the difficulty that we never know exactly the composition of the medium at the boundary; it is a mixture of the substances present in the sol and in the supernatant liquid. Therefore the mobility measured in most cases is of doubtful value.

Already HARDY in his work on globulins (7) made use of the outer liquid, formed during dialysis, as supernatant solution. Similarly KRUYT and his collaborators (22) have tried to get a homogeneous way of current by taking the ultrafiltrate as supernatant liquid. For some dilute sols it is really possible to obtain a filtrate of almost exactly the same conductivity as the sol, and in such cases the moving boundary method can be applied. In other cases the filtrate still has a much lower conductivity, for example for a 2.2%  $\text{As}_2\text{S}_3$ -sol the conductivity was  $16.15 \times 10^{-5}$  for the sol and  $6.21 \times 10^{-5}$  for the filtrate. KRUYT and VAN DER WILIGEN in this case obtained fairly good results by calculating the two different potential gradients in the sol and in the supernatant liquid from the conductivities, and making use of these two different values in calculating respectively the mobility from the downwards and the upwards migrating boundaries. This method is, of course, not exact; it is based upon the presumption that no mixing of the electrolytes on both sides of the boundaries by diffusion or ionic migration takes place during the time of experiment. This is of course true only for experiments requiring relatively short time, that is, in cases of great electrophoretic mobilities.

It is evident that in such cases when sols can be obtained electrolyte-free or of exactly known electrolyte content it is always possible by addition of a certain



amount of electrolytes to the sol and taking the supernatant liquid of the same electrolyte content as the mixture to obtain the same ionic medium on both sides of the boundary. The differences caused by ions bound by the colloid can be made negligible, if the electrolyte content is sufficiently high.

In fact, exact measurements of electrophoretic mobility are of value only in such cases where the electrolyte content is accurately known, on account of the big sensitivity of the mobility towards even very small changes in the composition of the medium.

Solutions of proteins form an example where this procedure is possible. The (qualitative) studies of MICHAELIS of the migration of proteins in buffer solutions and the quantitative measurements of SVEDBERG-SCOTT and SVEDBERG-TISELIUS are examples of this kind (see further below).

The moving boundary method has generally been applied only to coloured sols. In its application to ions of ordinary electrolytes, however, colourless solutions have also often been studied, since the very sharp boundaries obtained by the automatic sharpening processes mentioned above are easily observed on account of the difference in refractive index. As already mentioned the conditions can not be made such as to give these very sharp boundaries when we have to deal with colloids. The refractive index method for accurate measurements of concentration gradients worked out by LAMM (23) is applicable especially to diffuse boundaries and may probably be applied to electrophoresis measurements of uncoloured sols.

Some colloids may be made visible by the Tyndall light. Special arrangements for utilizing this in the observation of boundaries in electrophoresis are described by KRUYT (19) and by DUMANSKI and KNIGA (24). In this way the electrophoresis of starch, gum arabic, gelatin, casein, agar-agar, silicic and stannic acid and aluminium hydroxide could be observed.

The fluorescence emitted by proteins when exposed to ultraviolet light from a mercury lamp was made use of for the observation of protein electrophoresis by SVEDBERG and his collaborators JETTE and SCOTT (25, 26). Later, SVEDBERG and the author tried to use the short-wave ultraviolet absorption showed by all proteins for the same purpose (27). Light absorption methods have also been used throughout this work.

### The transference method.

The migration of a colloid may also be followed by analytical determination of the change in concentration in the neighbourhood of one of the electrodes. If in a solution containing  $a$  gram of a substance per cubic centimeter a quantity of  $m$  gram is transferred when  $Q$  coulomb is sent through the solution, if further the conductivity of this solution is  $\kappa$ , the mobility of the substance is obtained from the equation

$$u = \frac{m \times \kappa}{Q \times c} \quad (2)$$

Measurements by this method have been made by DUCLAUX (18), VARGA (28), WINTGEN (29), GREENBERG and SCHMIDT (30, 31), and others, even if they have not generally calculated the mobility from their measurements but related quantities («Kolloidäquivalent»).

No doubt the transference method from one point of view must be considered the least objectionable: The difficulties caused by the boundary anomalies are eliminated. Care has, of course, to be taken that no changes in colloid or ionic concentration take place in the middle portion of the apparatus. However, in order to get sufficient transference to obtain an accurately determinable increase in concentration at one electrode relatively large quantities of electricity have to be sent through the apparatus, and the products of electrolysis thus formed may easily reach the middle portion and cause changed mobility or even coagulation. At the electrodes coagulation almost always occurs and this causes difficulties in the analytical determination.

To eliminate this influence of electrode reactions MC BAIN and BOWDEN (32) and later PAULI and ENGEL (17) made use of a transference apparatus where the colloid was separated from the electrodes by a layer of an electrolyte solution. The PAULI-ENGEL apparatus is similar to a U-tube apparatus used in the moving boundary method, except that the migration is determined by determination of the change in colloid concentration in a part of the U-tube containing the boundary between two stopcocks.

Only few measurements with this apparatus have been published, but, judging from these, good results are obtained, at least for high mobilities.

One disadvantage of the transference method (as compared to the moving boundary method) of importance in work with colloids is connected with the fact that the former does not give any information about the uniformity of the migrating substance. We can never determine by this method if the observed mobility is the mean value of two or more differently migrating colloids' mobilities, or perhaps of a colloid and crystalloidal ions, or if it really represents an equal mobility for all the sol particles. In most cases, and perhaps especially for the »biocolloids»: proteins, carbohydrates, enzymes, antibodies and so on, this question is of considerable importance, and the determination of mobility of little value if we do not know whether it is due to a single substance or not.

The moving boundary method, on the other hand, furnishes a means of giving us some information about this question by the possibility it affords of observing the separation of the differently migrating substances at the boundary. Some attempts to apply the method in this direction will be related below.

### Previous work on proteins.

As far back as 1892 LINDER and PICTON, when studying the electrophoresis of colloids, also observed the migration of hemoglobin. The discovery of the amphoteric



behaviour of protein electrophoresis is, however, due to HARDY (7). In his work of 1899 he studied the migration of a boiled egg-white suspension and found that in acid solutions it migrated to the cathode and in alkaline solutions to the anode. He concluded therefore that at a certain acidity the egg-white suspension does not migrate at all, but must be »isoelectric» with the surrounding medium (33). In a later work (1905) previously referred to (7), the electrophoresis of serum globulin was studied quantitatively in the U-tube. The globulin was purified by reprecipitation and was dissolved in acid, alkali and salts to a more or less opalescent solution so that an observation of the boundaries was possible.

Since the moving boundary method could not be applied to clear solutions of uncoloured proteins, transference methods were used in the investigations following upon HARDY'S. These determinations were only qualitative and were made with the main purpose of finding the position of the isoelectric point for different native proteins. PAULI used an apparatus consisting of three beakers connected with two siphons for studying the migration of the serum proteins (34). Later an improved type of apparatus was constructed by LANDSTEINER and PAULI (35), consisting of a U-tube with stopcocks in each limb for the formation of the boundaries.

A great number of determinations of this kind have been made by MICHAELIS and his collaborators, who used an apparatus similar to that of LANDSTEINER and PAULI, but with the important improvement that reversible electrodes were introduced (36). The first determinations were made with enzymes, but later proteins were studied: serum albumin, oxyhemoglobin, gelatine and casein. The proteins were dissolved in buffer solutions of varying hydrogen ion concentrations, and the  $p_H$  were measured electrometrically. The  $p_H$  value, where no migration or a migration towards both sides was observed, was taken as the isoelectric point.

The results were discussed from the standpoint of the well-known MICHAELIS theory of the isoelectric point of an ampholyte, and it was found among other things that the isoelectric points determined by electrophoresis coincided with the points of minimum solubility of the proteins, in agreement with the theory and with the behaviour of amino acids also studied by MICHAELIS (see further below p. 83).

Quantitative transference determinations were made by GREENBERG and SCHMIDT (30) on casein, dissolved in alkali, but since conductivity measurements on the solutions studied were not performed, the mobility can not be calculated. Later GREENBERG (31) made transference determinations on fibrin in acid and alkali and from his measurements calculated the mobility.

An attempt to obtain the mobility of serum albumin in dilute hydrochloric acid by a combination of potentiometric determinations of ionic concentrations and conductivity measurements was made by PAULI and ODÉN (37), but since the assumption had to be made that the serum albumin chloride behaves like an ideal strong electrolyte the result must be considered as uncertain.

Quantitative determinations of electrophoresis in the neighbourhood of the isoelectric point are of course more difficult, on account of the small mobilities to

be observed. Such determinations were made by SVEDBERG and SCOTT (26) with egg albumin in buffer solutions of varying  $p_H$ , by taking photographs as related above of the migrating boundaries in the fluorescence light.

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The main purpose of the present investigation was to study the application of the moving boundary method for measurements of the electrophoresis of some comparatively well-defined proteins, and especially for the determination of their isoelectric points. Also attention was directed to the problem of the uniformity of the electrophoretic migration. For the realization of these purposes, it was found necessary to study the sources of error of the method, especially the boundary anomalies and the effects produced by the heat of current.

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## The apparatus and the method.

### General remarks.

The fluorescence method for observing protein electrophoresis referred to at the end of the preceding section has certain disadvantages. The time of exposure is inconveniently long (about 10 minutes in the work of SVEDBERG and SCOTT). A

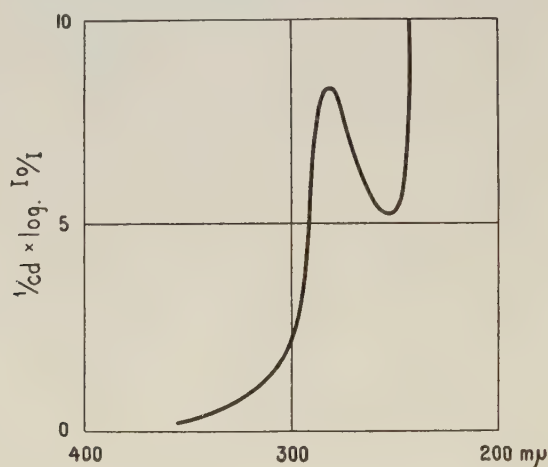


Fig. 2.

water thermostat cannot be used, since it is impossible to prevent the water from becoming fluorescent after a time, and therefore the heat produced by the current in the apparatus is more liable to give rise to convection currents. Moreover, it has not yet been proved that the fluorescence is really due to the protein itself and not to some partially adsorbed impurity. In any case the fluorescence of proteins seems to be a not very reproducible and constant property.

In the present work use will be made of the method of observation by ultraviolet light absorption used by SVEDBERG and the author in some preliminary measurements with egg albumin and in the work with the ultracentrifuge by SVEDBERG and his collaborators.

According to the light absorption measurements made by the above and other scientists, we know that all proteins investigated show a more or less strong light absorption in the ultraviolet region of wave length below 300  $m\mu$ . An example is

given in fig. 2 which shows the extinction coefficients  $\left(\frac{1}{cd} \times \log \frac{I_0}{I}\right)$  for egg albumin in salt-free solution, determined with the JUDD LEWIS sector spectrophotometer by SVEDBERG and the author.

A similar shape of the ultraviolet light absorption curve is obtained with the amino acids tyrosine and tryptophane.

Some proteins, such as hemoglobin, phycoerythrin, phycocyan, and (to a less extent however) hemocyanin, have strong light absorption in the visible region. They also show sufficient light absorption in the long-wave ultraviolet (below 400  $m\mu$ ) to make it possible to photograph the electrophoresis with light of these wavelengths.

The method used in the present work is the following. Photographs are taken of the position of the migrating boundaries in the U-tube, at certain time intervals, use being made of visible, long-wave or short-wave ultraviolet light. On the same plate as was used for the exposures of the boundaries a concentration-blackening scale is photographed. By microphotometric registration of the exposures, with the distances enlarged five times, a number of curves are obtained, representing the concentration distribution at the boundaries at different times. From the displacement of the curves with time, the mobility is calculated.

Partly on account of the imperfections in the method for obtaining the boundary and partly on account of the diffusion of the proteins studied, the boundaries were never quite sharp. Since most determinations of mobility were made in the neighbourhood of the isoelectric point and consequently the distances of migration to be measured were very small, it was difficult to obtain any accurate values by direct measurement on the plate, as was done in the preliminary work of SVEDBERG and the author (27). However, the mobilities of most colloids is higher and the diffusion lower than is the case with proteins under the conditions mentioned, and therefore direct measurement on the plate would probably in many cases be quite sufficient.

In this work, the microphotometric records have also been of value for other purposes than making the measurements more accurate. They have made it possible to study the boundary anomalies, and in some cases of anomalous migration, to calculate the mobility when this otherwise would not have been possible. With the aid of these records, some conclusions have also been drawn as regards the uniformity of the proteins studied.

### The apparatus.

Glass is not transparent enough for the short wave ultraviolet light used and therefore the U-tube had to be made of quartz. The first experiments of SVEDBERG and the author (27) were made with two ordinary tubes of clear, fused quartz. These tubes, however, were not uniform in diameter and had many bubbles and



other imperfections in the walls. All experiments in this paper were made with the U-tube in fig. 3, made by HERAEUS, Hanau. Two tubes of fused quartz »extra Blasenfrei» were ground cylindrically, polished inside and outside and afterwards molten to a short U-tube of quartz, with a special, funnel-shaped capillary in the bottom. The tubes were provided with two indices of black-nickeled brass, as shown in fig. 3. The index distance was 30.15 mm. The cross section area of the tubes was determined by filling each limb with water ( $16^{\circ}$ ) to the upper and the lower index and taking the difference in weight.

For one tube was found 2.301 gr. and 2.282 gr.; mean value 2.292 gr., for the other 2.289 gr. and 2.303 gr., mean value 2.296 gr. The cross section is therefore  $0.7615 \text{ cm}^2$ .

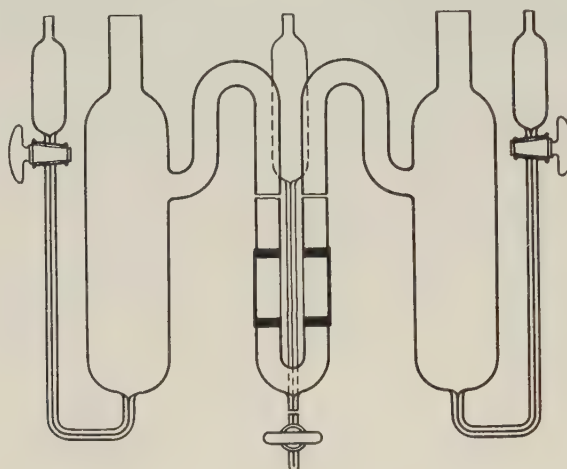


Fig. 3.

The capillary was connected with the protein container capillary by a vacuum rubber tubing. This container (of Jena glass) had a two way stopcock.

The electrode vessels were comparatively large in order to prevent the substances present or formed at the electrodes to reach the boundaries during an experiment. They were provided with containers for the electrode solution: saturated potassium chloride. They were made with narrow necks in order to make the free surface of solution as small as possible (see p. 45). The vessels were connected to the quartz U-tube with rubber tubing. The ends of the tubes connected were ground plane and brought into contact with each other under the rubber.

The different glass and quartz parts were mounted together on a black-nickeled brass plate with supports fitted to the thermostate, securing an exactly defined position for the U-tube. The plate served at the same time as a screen to cut out all light except that passing rectangular openings behind the limbs of the U-tube.

Even in many recent measurements of electrophoresis irreversible electrodes were used, but according to the experience of the author reversible electrodes are

quite indispensable. If, as really has occurred, the electrodes have been damaged and gas evolution sets in during the experiment this may cause an error of 20—30 % in the mobility.

The copper-copper sulphate and zinc-zinc sulphate electrodes described in previous work (26, 27) cannot be used in alkaline buffer solutions and furthermore have certain disadvantages in work with proteins, which are extremely sensitive to small amounts of the salts of these metals. Therefore silver-silver chloride electrodes in saturated potassium chloride were used throughout this investigation. They were prepared as follows: Two thin (0.1 mm.) silver strips, about  $25 \times 300$  mm. in size, were rolled to loose spirals and fused to silver wires. After being cleaned in nitric acid and washed with distilled water, the wires were covered with picein and the electrodes made anodes in a concentrated potassium chloride solution. A current of 10 milliamps was sent through for 3—4 days, after which time they were covered with a sufficiently thick layer of silver chloride. When in use, it was always so arranged, that one electrode was anode about the same length of time as it was cathode, and in this way they remain unchanged very long.

The use of reversible electrodes also has the advantage of giving a comparatively constant current through the apparatus.

The current was generally taken from a storage battery of 50 cells.

In most electrophoresis work the voltage on the apparatus is read off, and from this the potential gradient in the tube is calculated. This method is not very accurate, since, at least for low potentials, the polarisation potential at the electrodes interferes. The electrolytic migration in the apparatus during an experiment may also cause a change in the distribution of the potential drop. Attempts have been made to eliminate this difficulty by applying two additional electrodes at the ends of the U-tube (22), but the simplest method is to measure the current and not the voltage. If the current is  $i$  ampere, the conductivity of the solution at a certain place in the U-tube  $\kappa$ , the cross section here  $q$  cm<sup>2</sup>, then the potential gradient  $F$  volt/cm. at this place is given by the expression

$$F = \frac{i}{q \kappa}. \quad (3)$$

If the mobility is  $u$ , the distance of migration in the time  $t$  seconds is  $F \times u \times t = u \times \frac{i t}{q \kappa}$ . Therefore the most correct way would be to measure the quantity of electricity  $i t$  passed, as in a transference experiment. Since, if reversible electrodes are used,  $i$  is rather constant, the more convenient way of reading off  $i$  several times and taking the mean value for each time interval could be used.

The current intensity was read off on a milliammeter with the scale from 0 to 3 milliamps. It was calibrated at the beginning and the end of this work.



On account of the extreme sensitivity of the boundaries towards heat convection currents the temperature must be kept very constant during an experiment. An air thermostate is not sufficient on account of the heat produced by the current in the apparatus. A strongly stirred, well-regulated water thermostate was used, with temperature fluctuations less than  $0^{\circ}.01$ . To prevent vibrations, the stirrer, its motor and transmissions were detached from the thermostate, being carried by a special table, isolated from the floor by thick rubber cushions. The thermostate was provided with circular, plane parallel windows of HERAEUS' fused quartz »extra Blasenfrei«. The windows could easily be taken off for cleaning; they were attached to the thermostate by screw rings and rubber packings.

The thermostate was placed on a wood stand with foot screws for adjustment (T, fig. 4).

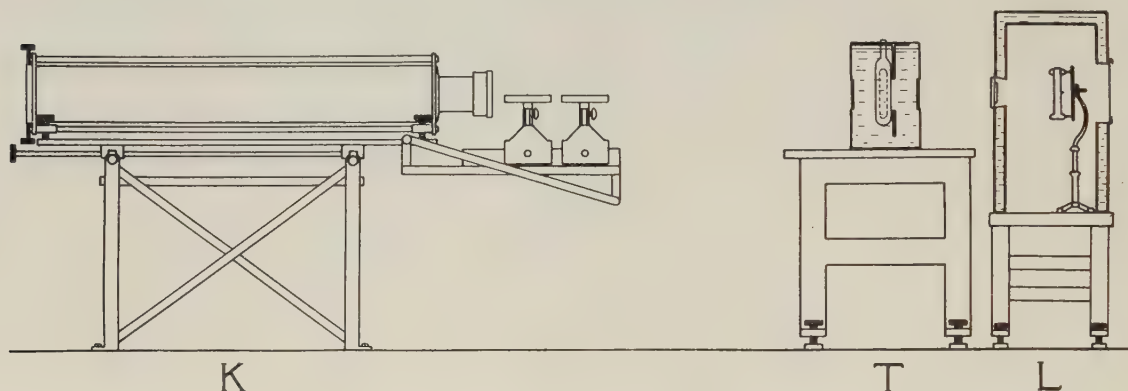


Fig. 4.

Behind this a quartz mercury lamp of HERAEUS' vertical type was placed in a water-cooled lamphouse (L, fig. 4). It was provided with a circular opening in front, covered by a screen of ground, fused quartz, 75 mm. in diameter. The lamp was fed from a storage battery and the voltage controlled by a voltmeter and kept constant within  $\pm 1$  volt by an adjustable resistance. Generally the lamp voltage was 120–130 volts. In some measurements in visible light a PHILIP's Argenta lamp of 500 watt was used instead of the mercury lamp.

Light filters were used in almost all experiments. For some of the measurements in the visible with phycoerythrin a WRATTEN K-filter (yellow) was placed in front of the objective. All the other measurements were made either in long-wave or in short-wave ultraviolet. Long-wave ultraviolet light (in experiments with phycoerythrin and phycocyan) was isolated by placing a CORNING nickel glass filter between the apparatus and the lamp, protected from the heat radiation of the latter by a water cuvette. This filter only lets through light of wave length  $366\text{ m}\mu$  and, to a lesser extent,  $334\text{ m}\mu$ . The former value is in the region of maximal absorption for phycoerythrin and phycocyan.

For the short wave region below 300  $m\mu$ , two filters with chlorine and bromine were used, according to PESKOW (38). Each filter consisted of a cylindrical glass tube, 80 mm. in length, with two stopcocks. On each end was cemented a brass ring, threaded to receive a brass cap with a circular opening. Between the cap and the end of the tube (which was ground plane) a window, made of plane parallel crystalline quartz, was inserted. Between the caps and the windows were rubber packings. The filters were filled with chlorine of atmospheric pressure and saturated bromine vapour. It was very difficult to make the bromine filter keep tight. The stopcocks and the ends of the glass tube were greased with a paste of moist phosphorus pentoxide, which was prevented from taking up further moisture from the air by an outer layer of vaseline. The vaseline itself must not come into contact with the bromine, since in that case after some time the windows become covered with a thin fog that strongly absorbs ultraviolet, probably through some interaction between the bromine and the vaseline. Still such a fog was often formed on the peripheral parts of the windows and gave rise to uneven distribution of light over the image. The chlorine filter, on the contrary, kept clear, and could be greased with vaseline without any trouble.

It is possible by means of these filters to cut out the light of wave lengths above about 280  $m\mu$ . Of course, visible light in yellow and longer wave lengths is let through, but if ordinary plates (not colour-sensitized) are used, this does not interfere. This could easily be controlled by taking two photographs in the light of a mercury lamp with these filters, one with, and one without a glass plate in front of the camera lens. The time of exposure was sufficient to give a strong blackening on the plate when the glass was left out. When the glass was put in, all short-wave ultraviolet was cut out and only visible light let through. No trace of blackening could be observed even if the plate was exposed 3 times longer than usual.

The focal length of the camera objective must be taken very long to avoid parallax. The camera (K fig. 4) consisted of a front and a back piece of brass, kept together with four long brass rods, and connected by a tube of thick, black cardboard. It was provided with four foot-screws for adjustment. For work in the visible and long-wave ultraviolet let through by the nickel glass filter a glass objective could be used: a DALLMEYER objective of 615 mm. focal length. The fixed distance between the objective and the plate was chosen so as to give an image of nearly natural size. The thermostate with the electrophoresis apparatus was moved on its table until a sharp image of the tube was obtained in the camera. The ratio in size between the image and the tube was 0.995. The chromatic correction of the objective was sufficient good to make unnecessary a special adjustment for long-wave ultraviolet work.

For work in the short-wave ultraviolet a quartz lens was used of 68 cm. focal distance for visible light, that is 63 cm. for ultraviolet of  $\lambda = 253 m\mu$ . It was biconvex for diminished spherical aberration. Like the DALLMEYER objective the



lens was placed at a distance from the plate equal to double the focal length. Consequently the distance between the apparatus and the camera had to be increased somewhat (36 mm.). Therefore holes for the camera foot-screws were made in the iron stand, carrying the camera, corresponding to the two different positions used. A change in the optics from visible to short-wave ultraviolet could in this way be conveniently made.

The ratio in size between the image and the tube for the quartz lens was 0.998. The index distance on the tube and on the image was measured with a comparator.

In the centre of the back part of the camera was a rectangular opening of size  $7.5 \times 35$  mm. Immediately behind this opening was the movable plateholder. Each image on the plate was therefore of the size  $7.5 \times 35$  mm. By moving the plateholder a certain distance after each exposure, twenty such exposures could be taken on a  $9 \times 12$  cm. plate (after ten exposures had been taken, the plate is turned  $180^\circ$  in the plateholder).

Each time two exposures had to be taken, one of each limb of the U-tube, for which reason the camera must be horizontally movable, perpendicular to the optical axis. An iron frame, carrying the camera, was therefore mounted on four saddles, movable by gears on cylindrical iron rods as shown in fig. 4. The movable frame also carried the filters on an optical bench.

The shutter was also placed here.

### The procedure.

The solutions to be used in an experiment were always prepared from boiled, distilled water. If the water is not air-free, disturbing bubbles may appear in the U-tube during the experiment.

The acetate buffer solutions were prepared from standard sodium and barium hydroxide and acetic acid solutions, and not from acetate. Sodium acetate solutions after some time always contain a certain amount of mould.

The phosphate buffers were made of 0.2 m solutions of  $\text{KH}_2\text{PO}_4$  and  $\text{Na}_2\text{HPO}_4$ , KAHLBAUM's »Zu Enzymstudien, nach SØRENSEN». The citric acid was also KAHLBAUM's »Zu Enzymstudien».

The potassium chloride used was three times recrystallized KAHLBAUM's »Zur Analyse». (The potassium chloride used as electrode solution was not recrystallized.)

Generally 500 cc. supernatant solution and 50 or 25 cc. protein solution were prepared for each experiment.

After being cleaned, the apparatus was filled to a certain height with supernatant solution and the protein and potassium chloride containers with their respective solutions. The apparatus was placed in the thermostat with all stopcocks

closed, and the electrodes connected to the switches of the battery. It was left for at least one hour to attain constant temperature. The thermostat was adjusted to 20°.0 in all experiments described in this work.

An experiment was started by opening the two stopcocks for the electrode solution. After a few minutes the bottom parts of the electrode vessels were filled with potassium chloride solution, and the apparatus was left for about 5 minutes, so that the liquid might come at rest again. If the quantities of the solutions were chosen correctly, the levels of the solution should now just reach the bottom of the tubes forming the top of the electrode vessels (fig. 3). If not, the flow of the protein may be not quite symmetrical since the two electrode vessels are not exactly identical except in the two top tubes, and this results in a difference in position between the two boundaries in the first exposure.

The electrode tubes were now closed with loose cotton plugs (to prevent evaporation during the experiment) and the stopcock of the protein container was opened cautiously, to avoid vibrations. This was done with a glass rod with a piece of rubber on the end. During the first minutes the protein solution was allowed to flow by its own pressure, but then the container was connected to a pressure flask with a fine rubber tubing. In this flask the air pressure could be regulated by a sort of water manometer arrangement. The rate of flow of the protein solution could be regulated in a reproducible and convenient way, without touching the electrophoresis apparatus, by changing the air pressure in the flask. If the boundary for some reason became unsatisfactory, the protein could conveniently be sucked back into the container again. In the ordinary construction of electrophoresis apparatus where only the difference in levels of the two liquids is the driving force for the flow, the protein container must be placed inconveniently high, and the speed of flow is greater at the beginning than at the end — the contrary of what it should be.

The formation of a sharp boundary is of course a very important condition for obtaining good results. In solutions of concentration 1% or higher it is fairly easy to get good results with this arrangement in a time of flow of about half an hour or even less. However, the concentration of protein cannot be chosen at will. If the tube thickness is given, the right contrast in the photographic image is obtained only for a very limited range of concentration. The quartz U-tube described above had an inside diameter of 9.9 mm. and for this thickness of layer protein concentrations of 0.1—0.4% were used. To be able to use higher concentrations one must take narrower tubes. Experiments were made with an apparatus with a glass tube of 3 mm. inside diameter, with 1% solutions, but the boundaries did not become better, rather the contrary. This is likely due to the fact that the retarding influence of the walls on the flow — which is probably the cause of the blurring of the boundary — increases very rapidly when the diameter is diminished. On the other hand the use of wide tubes is inadvisable on account of the diffi-



culties to cool them off properly, which may easily give rise to convection currents especially since in this case dilute solutions have to be used.

Boundaries, that still looked satisfactory to the eye, often showed in the photometric records the kind of deformation demonstrated in fig. 5, the upper curve (lower curve normal). This kind of deformation was especially marked if the speed of flow was high. On the other hand, a very slow boundary formation process gives boundaries too much blurred by diffusion. Experiments were made to work out the best conditions, and for a 0.2% solution fairly good results were obtained with 45 min. total time of flow and a very low speed during the first 5—10 minutes, after which the flow was gradually increased.

When the boundaries had reached the middle between the indices, the stopcock was closed and the apparatus was left for about 10 minutes so that the boundaries

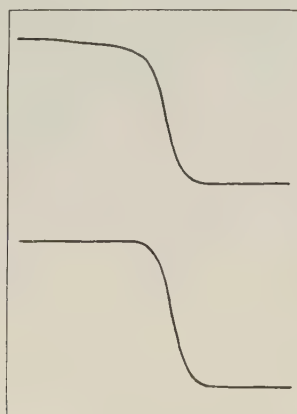


Fig. 5.

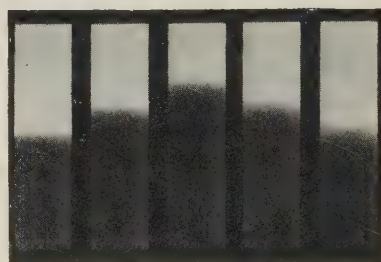


Fig 6.

might come to complete equilibrium. In the meantime test exposures were taken on a plate  $4\frac{1}{2} \times 6$  cm., to try out the best time of exposure.

The lamp was turned on at least 15 minutes before the first exposure. The flow of cooling water for the lamphouse could be regulated with a tap, provided with a scale and a pointer, which was always kept in the same position. Since the lamphouse was provided with a quartz window, it was never necessary to open it during the experiment. The cooling of the mercury lamp was therefore always constant.

In the earlier experiments with phycoerythrin visible light from a 500 W Argenta lamp was used with a WRATTEN K-filter and Ilford Screened Chromatic plates. Later, only short-wave and long-wave ultraviolet was used in all experiments, with Imperial Process plates. These plates give much less fog, and though they are considerably slower than ordinary plates in the visible, their sensitivity in ultraviolet is about the same.

The diaphragm used was F:40 or F:60 and the time of exposure varied from 10 to 60 seconds.

The exposures of the left tube were taken on fields nr. 1—5 of the plate, those of the right tube on nr. 6—10, starting at nr. 1 and nr. 6. After the first exposures the current was closed, and the strength of current read off repeatedly. Generally it varied little (see the tables below). The current was left on for a time of from 15 minutes to several hours, depending upon the speed of migration, then exposures were taken on field 2 and 7.

After this, the boundaries were generally allowed to migrate in the same direction for one more time interval of about the same length. After exposures on 3 and 8, the current was reversed and the boundaries allowed to move back again. Generally 3—5 exposures were taken, corresponding to 2—4 time intervals giving 4—8 values of the mobility. Fig. 6, corresponding to exp. 1 (see p. 64) with phycocyan shows such a series of exposures (right tube).

To obtain accurate values, as long distances of migration as possible should be used. By using long time for the experiment, on the other hand, the blurring of the boundaries by diffusion will diminish the accuracy and the diffusion and ionic migration of the electrode solution may reach the boundary (see further p. 42). Generally the total experiment did not last more than 8 hours, only in a few exceptional cases was it stretched out over a period of 24 hours, the last time interval being about 12 hours taken in the night.

When an experiment had been completed, the electrophoresis apparatus was taken out, emptied and washed with distilled water. The electrode tubes were taken away, only the U-tube being left on the stand. This U-tube was now successively filled with mostly five different concentrations of the protein used in the experiment:  $c$  (= protein solution undiluted),  $3c/4$ ,  $c/2$ ,  $c/4$ , and 0 (= pure buffer solution). Photographs were taken of one limb, under the same conditions as in the experiment. These photographs were taken on the same plate as had been used in the experiment after it had been turned  $180^\circ$  in the plateholder.

In this way, after development, a concentration scale was obtained on the plate, which gives the relation between the concentration in the tube and the blackening on the photographic plate.

This procedure is similar to that used in the work with the ultracentrifuge (39).

In most cases an ordinary metol-hydrochinone developer was used, with a time of development of about 1 minute at room temperature. In some cases metol or hydrochinone was used alone to reduce resp. increase the contrasts.

The plates were photometered with a selfregistering SIEGBAHN microphotometer (40). The microphotometer had a resolving power of 0.05 mm. It was adjusted for exactly 5 times enlargement of distances (controlled repeatedly by the registering of a ZEISS millimeter scale). The original plate was moved with a speed of about 2.5 mm. per minute in the registration; the concentration scale images were, however, taken with higher speed. Fig. 7 gives a typical record obtained in this



way. From these records curves are drawn on transparent millimeter paper, so that the lines corresponding to the index edge (to the left in fig. 7) coincide exactly. These curves, of which numerous examples will be found below, form the basis for the calculation of mobility.

If the concentration of the protein solution has not too high a value (this depending upon the light absorption coefficient of the protein) one can always, by choosing a suitable time of exposure and development obtain a concentration scale, that gives an almost linear relation between concentration and galvanometer deflection on the microphotometric records, with the concentrations zero and  $c$  nearly corresponding to the extremes of galvanometer deflection on the record plate (39).

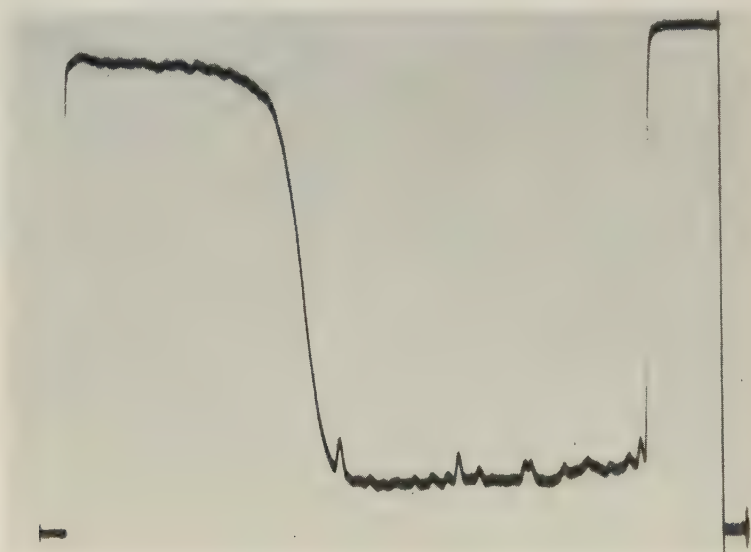


Fig. 7.

This gives of course the best possible reproduction of the concentration distribution in the tube. If the protein concentration is too high, a concentration-deflection curve of more or less pronounced S-shape is obtained, provided that the time of exposure is increased sufficiently to let the concentration  $c/2$  correspond to about half the maximal galvanometer deflection. Consequently, in this case, the reproduction of the low and the high concentration parts of the tube is poor, that of the medium concentrations, however, very good.

If the concentration is too low, the linear relation is obtained, if the time of exposure is somewhat shorter, but the slope of the line is small, and therefore the reproduction of the whole concentration range not accurate.

By using soft or hard developers the effect of errors of this kind may be reduced, and the available range of protein concentrations increased. This has

been of special value in the present work, where only one thickness of solution layer could be used.

In this work it was preferred to use rather high concentrations, since concentrated solutions give sharper and more stable boundaries.

### $P_H$ measurements.

For the  $p_H$  measurements only the hydrogen electrode was used. The measurements were made at room temperature, that was read off each time (generally  $+18^\circ$ ); no thermostat was used.

The potentiometer was a LEEDS and NORTROP Type K instrument, the galvanometer (SIEMENS & HALSKE) had 300 ohms system resistance. The hydrogen was taken from a tank and was purified from oxygen in an electrically heated quartz tube containing pieces of copper wire as catalyzer. It was washed with a solution of mercury chloride and was then led to the electrode vessel.

The calomel cells contained 0.1 normal potassium chloride. The calomel was prepared electrolytically according to LIPSCOMB and HULETT (41), the mercury was purified by two vacuum distillations after having been washed several times with a mixture of dilute nitric acid and mercurous nitrate. The cells were refilled repeatedly during the work.

One difficulty in  $p_H$  determinations of protein solutions is connected with their strong tendency to foam. This often makes the application of the ordinary »bubbling-through» type electrode vessels impossible. Therefore the type of vessel shown in fig. 8 was used. Here the hydrogen passed over the solution, but the vessel was closed several times and rotated with an electric motor for about 3—5 minutes each time. The measurements were made without taking away the vessel from its axis of rotation. The shaking method of saturating the electrode solution with hydrogen was first used by HASSELBALCH (42).

Following the standardization suggested by SØRENSEN and LINDERSTRØM-LANG (43), and also adopted by CLARK, the  $p_H$  was calculated from the voltage  $\pi$  by the equation

$$p_H = \frac{\pi - 0.3380}{0.05773} \quad (4)$$

at  $18^\circ$ . At  $15^\circ$  0.3382 and at  $20^\circ$  0.3379 is put in instead of 0.3380. Corrections for the vapour pressure of water and barometric pressure were applied, according to the tables of CLARK (44).

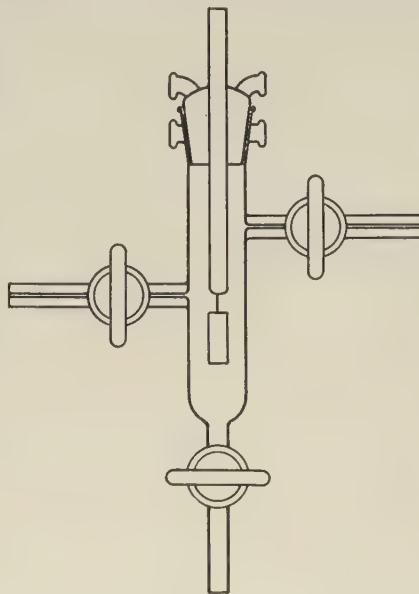


Fig. 8,



In the course of the work the arrangement was repeatedly checked against the mixture of 0.01 n hydrochloric acid and 0.09 n potassium chloride recommended by SØRENSEN and LINDERSTRØM-LANG. The BJERRUM extrapolation method for eliminating liquid junction potentials was used in this case. The value ascribed to this combination, 0.4556 volts, was mostly reached within  $\pm 0.0005$  volts. If not, the calomel cells were refilled.

All the other measurements were made with saturated potassium chloride solution as bridge between the electrodes.

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## A discussion of the method and its sources of error.

### The equation of the boundary migration.

Let us consider a vertical tube of  $1 \text{ cm}^2$  area (fig. 9) containing an electrolyte solution. The original concentration may vary with the height in an arbitrary way. A current is sent through the tube in the  $x$  direction. We consider a thin horizontal layer of thickness  $dx$  at a great distance from the electrodes. Let  $c$  be the concentration of an arbitrary ion  $i$  with the mobility  $u$  (with the sign equal to the sign of charge of the ion),  $t$  the time,  $x$  the distance and  $F$  the field intensity = the negative value of the potential gradient. The amount of ions passing into the layer in the time  $dt$  is  $u' \times F' \times c' \times dt$ , and at the same time  $u'' \times F'' \times c'' \times dt$  is brought out of it (see fig. 9). The concentration change therefore is

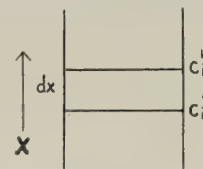


Fig. 9.

$$dc = \frac{u' F' c' - u'' F'' c''}{dx} dt, \quad (5)$$

and therefore

$$\frac{\partial c}{\partial t} = - \frac{\partial (u F c)}{\partial x}, \quad (6)$$

an equation obtained by KOHLRAUSCH (15) and by WEBER (16).

Suppose now, that besides the ion  $i$  other ions are present and at least some of these in sufficiently high concentrations, constant throughout the tube, to make  $u$  and  $F$  independent of  $x$ . If  $u$  is also assumed to be independent of  $c$ , we obtain

$$\frac{\partial c}{\partial t} = - u F \frac{\partial c}{\partial x}. \quad (7)$$

The solution of this partial differential equation is evidently

$$c = f(x - F u t), \quad (8)$$

where  $f$  is an arbitrary function, determined by the concentration distribution at the start ( $t = 0$ ). The equation says that this distribution remains unchanged



during the experiment, only that all concentrations are moved with the constant velocity  $Fu$ .

In the simple case mentioned, the equation can easily be generalized to take the diffusion also into account. If in the deduction above we apply Fick's law we get for the transport by diffusion the expressions  $-D \times \left(\frac{\partial c}{\partial x}\right)' \times dt$  and  $-D \times \left(\frac{\partial c}{\partial x}\right)'' \times dt$  to be added to the terms corresponding to the ionic migration. ( $D$  is the diffusion constant.) Therefore we obtain

$$\frac{\partial c}{\partial t} = -uF \frac{\partial c}{\partial x} - D \frac{\partial^2 c}{\partial x^2}. \quad (9)$$

If instead of the solution above we try the expression

$$c = f_t(x - uFt), \quad (10)$$

where the expression for the function itself is a function of  $t$ , we find that the equation is satisfied if

$$\frac{\partial f_t}{\partial t} = -D \frac{\partial^2 f_t}{\partial x^2}, \quad (11)$$

where the derivative  $\frac{\partial f_t}{\partial t}$  corresponds to the last mentioned variation only. This means that the diffusion is simply superimposed on the electrophoretic migration, since the equation is identical with the differential equation for the diffusion at a resting boundary.

The above equations are valid only for one single migrating substance. If there are more than one and the mobilities of these substances are independent of each other, their different movements are, of course, superimposed on one another. If we have only one substance, consisting of several forms with different migration velocities in equilibrium with each other, it will generally migrate as one uniform substance. Suppose that in a certain medium the substance contains the fraction  $\alpha_1$  of the form with the mobility  $u_1$ ,  $\alpha_2$  with  $u_2$  and so on, then the observed mobility of the uniformly migrating substance is given by

$$u = \alpha_1 u_1 + \alpha_2 u_2 + \cdots + \alpha_n u_n. \quad (12)$$

This may be proved by statistical reasoning: During the time for the total migration  $t$  an arbitrary particle exists in the form 1 for a total time  $\alpha_1 t$ , in the form 2 for the total time  $\alpha_2 t$ , and so on. Any particle therefore migrates the distance

$$\alpha_1 t u_1 + \alpha_2 t u_2 + \cdots + \alpha_n t u_n \quad (13)$$

from which the above expression follows.

An example of this kind of migration would be the electrophoresis of an ampholyte HAOH containing  $\text{HA}^+$  and  $\text{AOH}^-$  ions, in a medium of constant composition.

Of course, if the average time of existence of a particle in a certain form is not very short as compared to the time of migration  $t$ , this way of reasoning is not correct and we may expect a spreading out of the boundary.

### The boundary anomalies.

If in the equation

$$\frac{\partial c}{\partial t} = - \frac{\partial (u F c)}{\partial x} \quad (14)$$

$u$  und  $F$  can not be considered independent of  $x$ , as in the instance dicussed above, we have the case of boundary anomalies. If the equation is written

$$\frac{\partial c}{\partial t} = - u F \frac{\partial c}{\partial x} - u c \frac{\partial F}{\partial x} - F c \frac{\partial u}{\partial x}, \quad (15)$$

it is more easily compared to eq. (7) above.

The nature of the effects to be expected in such a case are easily realised from a simple example. Suppose that only  $u$  and not  $F$  varies with  $x$  and that  $u$  is a linear function of  $x$ :  $u = Ax + B$ . We obtain

$$\frac{\partial c}{\partial t} = - (Ax + B) F \frac{\partial c}{\partial x} - F A c. \quad (16)$$

This equation is easily solved. The equivalent system of differential equations is

$$\frac{dx}{(Ax + B)F} = dt = - \frac{dc}{FAc} \quad (A \neq 0) \quad (17)$$

which gives the integrals

$$c = \text{const}_1 \times e^{-FA t} \quad (18)$$

and

$$Ax + B = \text{const}_2 \times e^{FA t} \quad (19)$$

whereas

$$c = e^{-FA t} \text{ f } [e^{-FA t} (Ax + B)] \quad (20)$$

is the solution, where  $f$ , the arbitrary function, is determined by the concentration distribution at the beginning ( $t=0$ ):

$$c = f[(Ax + B)]. \quad (21)$$

From the last two equations it is found that if  $F$  and  $A$  have the same sign, the boundary is gradually spread out with increasing  $t$ , whereas if  $F$  and  $A$  have opposite signs the boundary is sharpened. This may be proved by plotting the corresponding curves, but it is immediately realised if we observe that the exponential factors act as if they changed the scale for  $c$  and the independent variable ( $Ax + B$ )

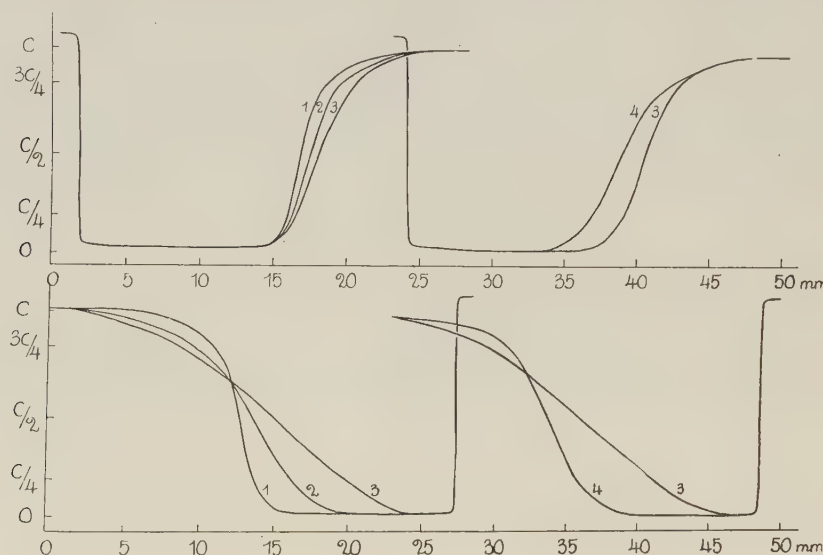


Fig. 10.

in the direction of decreasing ordinates and increasing abscissæ (first case) or increasing ordinates and decreasing abscissæ (second case). We get the rule that migration into a region of increasing mobility causes a spreading out, whereas migration into a region of decreasing mobility causes a sharpening of the boundary. Of course the sharpening effect may be, partially or entirely, masked by the diffusion. An example demonstrating effects of this kind is given in fig. 10. It represents the photometric records from an experiment with egg albumin, dissolved in 0.02 n potassium chloride, with the same salt in the same concentration as supernatant liquid. The conductivities of the two solutions are practically the same (see table V below) and therefore  $F$  is constant. However, the  $p_H$  of the protein-potassium chloride mixture is 4.81, whereas the  $p_H$  of the supernatant solution was some place in the neighbourhood of 6 (could not be measured exactly). Since  $u$  increases with



$p_H$  we have  $\frac{\partial u}{\partial x} > 0$  in both tubes and get therefore sharpening effect in one tube and blurring in the other. When the current is reversed the effects are reversed too.

Further data concerning this experiment are found in table V p. 36 below. It is seen immediately from fig. 10 that a calculation of the mobility from the boundary movement only (that is, from the movement of the  $c/2$  level) would give widely differing values for the two boundaries (except for the first time interval) and is of course not justified.

Similar effects are obtained if there is a gradient in  $F$  and  $u$  is constant, as may be the case if the two solutions have different conductivities.

When studying electrophoresis with the moving boundary method the cause of boundary anomalies is, of course, generally a difference in composition between the colloid and the supernatant solution, giving rise to variations in  $u$  or in  $F$ . In such cases we cannot generally expect to know sufficient about the relations between  $u$ ,  $F$ , and  $x$  to be able to apply the above equations for the calculation of the mobility from the observed changes of concentration distribution with time. Moreover, if the conditions are such as to give rise to boundary anomalies, further complications may occur, as will be mentioned below (p. 37). For experimental work, therefore, it is generally necessary to choose the conditions so that the anomalies are depressed as much as possible. As regards the magnitude of the error in the mobility caused by the boundary anomalies only, it may be stated that it can not exceed the difference  $\Delta u F$  between the migration velocities of the colloid in the two media in question, since at any level in the tube the colloid migrates with a velocity somewhere between those two extremes.

It should be observed that boundary anomalies are easily distinguished from those migration effects which are a result of an inhomogeneity of the migrating substance with regard to mobility (see pp. 88—100). The former effects are always of opposite sign at the two boundaries, sharpening at one boundary is always accompanied by blurring at the other, whereas in the case of inhomogeneous migration both boundaries behave in the same manner.

### The depression of the boundary anomalies.

It is evident that the boundary anomalies are completely eliminated if the electrolyte composition of the colloid and the supernatant liquid is exactly the same and sufficiently high to make the contribution to conductivity of the colloid substance itself of no importance. How high this concentration must be depends upon the amount of electrolyte bound by the colloid. It is of considerable importance to determine the smallest possible quantity necessary for this purpose, since the use of well-conducting media greatly increase the difficulties in electrophoresis work and may make measurements impossible, on account of the heat effects (p. 40).

Of course the media must be equal for all ions and other substance determining the mobility, as was demonstrated in the experiment in potassium chloride just described.

Since the mobility of proteins is mainly dependent upon the  $p_H$  value of the medium, the simplest method of obtaining a homogeneous way of current is to use buffered solutions, of sufficient concentration and of equal composition in both media. In order to find out how high concentrations are necessary in a typical case (which, of course, mainly depends upon the concentration and electrolyte combining capacity of the protein studied) a series of electrophoresis experiments were made with egg albumin solutions of 0.35 % conc. in sodium acetate — acetic acid mixtures of varying sodium acetate concentrations (0.002 n—0.02 n) and varying  $p_H$  (at  $p_H$  4.2 and 5.1). It was found that strong boundary anomalies occurred in the interval

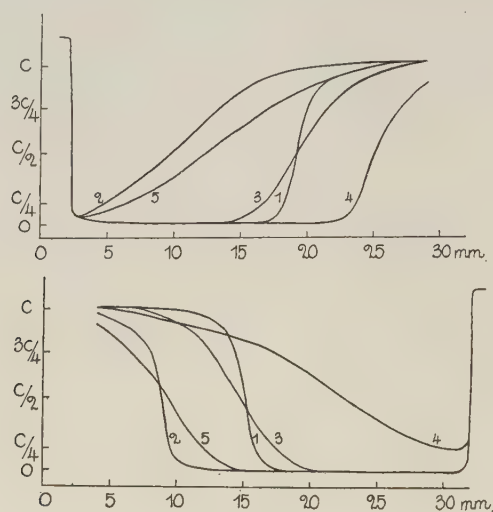


Fig. 11.

0.002 n—0.005 n acetate concentration, whereas at 0.01 n acetate concentration the anomalies were hardly detectable, at least not in the solutions of  $p_H$  4.2.

A typical experiment from this series, exhibiting very strong boundary anomalies, is demonstrated in the curves of fig. 11. The egg albumin concentration was 0.35 %, and the solutions contained 0.002 n sodium acetate and 0.0068 n acetic acid. The  $p_H$  of the protein-buffer mixture was 4.30, of the buffer 4.20, the conductivities were  $\kappa = 1.92 \times 10^{-4}$  and  $\kappa = 1.83 \times 10^{-4}$  respectively. The migration was cathodic. The strength of current was 0.365 milliamps and the time intervals 25, 25, 90, and 160 minutes.

The magnitude of the differences in  $p_H$  and conductivity for the solutions used may be seen from the following two tables II und III.

Table II.

Conductivity differences between buffer and protein-buffer mixtures for acetate-acetic acid mixtures 1:3.4 and 1:0.4. Protein concentration 0.35 %. Conductivity of 3.60 %, electrodyalized egg albumin stock solution  $2.9 \times 10^{-5}$ . Conductivity of water  $9 \times 10^{-6}$ .

| Acetic acid concentration | Sodium acetate concentration | $\kappa$ in protein   | $\kappa$ in buffer    | Difference |
|---------------------------|------------------------------|-----------------------|-----------------------|------------|
| 0.068 n                   | 0.02 n                       | $1.46 \times 10^{-3}$ | $1.46 \times 10^{-3}$ | <1 %       |
| 0.034 n                   | 0.01 n                       | $7.86 \times 10^{-4}$ | $7.78 \times 10^{-4}$ | +1 %       |
| 0.017 n                   | 0.005 n                      | $4.21 \times 10^{-4}$ | $4.13 \times 10^{-4}$ | +2 %       |
| 0.0068 n                  | 0.002 n                      | $1.92 \times 10^{-4}$ | $1.83 \times 10^{-4}$ | +4.5 %     |
| 0.008 n                   | 0.02 n                       | $1.43 \times 10^{-3}$ | $1.44 \times 10^{-3}$ | <1 %       |
| 0.004 n                   | 0.01 n                       | $7.41 \times 10^{-4}$ | $7.50 \times 10^{-4}$ | -1.2 %     |
| 0.002 n                   | 0.005 n                      | $3.86 \times 10^{-4}$ | $3.91 \times 10^{-4}$ | -1.3 %     |
| 0.0008 n                  | 0.002 n                      | $1.60 \times 10^{-4}$ | $1.63 \times 10^{-4}$ | -1.9 %     |

Table III.

$P_H$  differences between buffer and protein-buffer mixtures for acetate-acetic acid mixtures 1:3.4 and 1:0.4. Protein concentration 0.35 %.

| Acetic acid concentration | Sodium acetate concentration | $p_H$ in protein | $p_H$ in buffer | Difference |
|---------------------------|------------------------------|------------------|-----------------|------------|
| 0.068 n                   | 0.02 n                       | 4.14             | 4.14            | +0.00      |
| 0.034 n                   | 0.01 n                       | 4.17             | 4.16            | +0.01      |
| 0.017 n                   | 0.005 n                      | 4.22             | 4.18            | +0.04      |
| 0.0068 n                  | 0.002 n                      | 4.30             | 4.20            | +0.10      |
| 0.008 n                   | 0.02 n                       | 5.07             | 5.08            | -0.01      |
| 0.004 n                   | 0.01 n                       | 5.06             | 5.09            | -0.03      |
| 0.002 n                   | 0.005 n                      | 5.05             | 5.12            | -0.07      |
| 0.0008 n                  | 0.002 n                      | 5.02             | 5.17            | -0.15      |

A difference in  $p_H$  of 0.01 corresponds to a difference of about  $0.1 \times 10^{-5}$  in mobility for egg albumin.

Evidently the use of a buffer concentration of 0.02 n must secure an elimination of the boundary anomalies, in agreement with the results of the electrophoresis



experiments just mentioned. Also with the other proteins investigated most experiments in acetate buffer were therefore performed at this concentration.

Electrophoresis experiments may of course also be performed in unbuffered solutions. For the elimination of the boundary anomalies the following considerations are of importance.

A protein solution of a certain acidity is generally not in equilibrium with a solution of the same acidity, free from protein. The free acid will have a tendency to diffuse into the protein solution and the equilibrium at last reached is characterized by a certain difference in acidity between the two solutions, the magnitude of which depends upon the charge of the protein and the salt content of the solutions. This phenomenon is the well-known DONNAN equilibrium (45), which was studied experimentally and theoretically by DONNAN and collaborators and also by LOEB (46), BJERRUM (47), and RINDE (48).

According to the theory the presence of the colloid causes an unequal distribution of the ions present by the establishment of a potential difference, the so called membrane potential, between the two solutions. If this potential difference is called  $\varphi$ , if further  $c'_1, c'_2, c'_3, \dots$  mean the concentrations of the different ions in the colloid and  $c''_1, c''_2, c''_3, \dots$  the corresponding concentrations in the other solution (in equivalents), and if  $E$  is the charge of the colloid in equivalents per unit of volume, and  $R$ ,  $T$  and  $F$  are the gas constant, the absolute temperature, and the charge of an equivalent respectively, if further  $n$  is the valency, the condition of equilibrium is given by the following equations

$$\varphi = \frac{RT}{n_1 F} \ln \frac{c'_1}{c''_1} = \frac{RT}{n_2 F} \ln \frac{c'_2}{c''_2} = \frac{RT}{n_3 F} \ln \frac{c'_3}{c''_3} = \dots$$

$$E = - \sum c' n. \quad (22)$$

It has been emphasized by DONNAN himself that the presence of a membrane is of course not necessary for the establishment of the equilibrium. On account of the general thermodynamical basis of the above equations one must assume that in any case when a colloid is in contact with an electrolyte solution there is a tendency of the electrolytes to diffuse so that the equilibrium condition is fulfilled and, if this condition was fulfilled from the beginning, no diffusion of electrolytes will take place. In fact, the author (49) showed that the DONNAN equilibrium plays an important rôle in the sedimentation equilibrium of a charged substance in the ultracentrifuge.

We may therefore apply DONNAN's theory to find out how the conditions must be chosen at the boundary in an electrophoresis experiment in order to maintain an equal acidity on both sides of the boundary. It is seen from the equations above that  $c'_1 = c''_1$ ,  $c'_2 = c''_2$ , and so on only if the electrolyte content is high. How high it must be can be estimated with the theory.

Suppose that only one (monovalent) electrolyte  $A B$  is present. We obtain

$$\varphi = \frac{R T}{F} \ln \frac{c_A''}{c_A'} = - \frac{R T}{F} \ln \frac{c_B''}{c_B'} \quad (23)$$

$$E = c_B' - c_A'.$$

From the first equation follows

$$c_A' c_B' = c_A'' c_B'' = c^2 \quad (24)$$

where  $c$  is the electrolyte content outside the membrane:  $c_A'' = c_B'' = c$ . This gives in the second equation

$$(c_B')^2 - E c_B' = c^2 \quad (25)$$

and therefore

$$c_B' = \frac{E}{2} + \sqrt{\frac{E^2 + 4 c^2}{4}} \quad (26)$$

which is the DONNAN equation for the influence of salts on the equilibrium.

Judging from the amount of acid bound by egg albumin (79) a value for  $E$  of about  $20 \times 10^{-5}$  equivalents per gram of protein at a  $p_H$  distance of 1.0 from the isoelectric point may be assumed. For a 0.4 % solution at a  $p_H$  distance of  $\pm 0.5$  this makes  $40 \times 10^{-5}$  eq. per litre. Using this value we obtain

$$\frac{c_B'}{c} = \frac{4 \times 10^{-4} + \sqrt{16 \times 10^{-8} + 4 c^2}}{2 c}. \quad (27)$$

By inserting varying values of  $c$  in this expression we obtain the following table.

Table IV.

Calculated values of the depression of DONNAN effects by a mono-monovalent salt.

| Electrolyte concentration<br>$c$ | $c_B'/c$ | $\log c_B' - \log c$ |
|----------------------------------|----------|----------------------|
| 10 <sup>-4</sup>                 | 4.2      | 0.62                 |
| 10 <sup>-3</sup>                 | 1.2      | 0.08                 |
| 10 <sup>-2</sup>                 | 1.02     | 0.009                |

If we consider for example hydrogen ions the third column gives the calculated  $p_H$ -difference between the two solutions for the different electrolyte concentrations.

Under the conditions mentioned we should therefore expect a salt concentra-

tion of about  $10^{-2}$  sufficient to secure the stability of a condition with equal  $p_H$  on both sides of the boundary.

This stability is evidently secured in the experiments in buffered solutions, at the buffer concentrations that were used. If experiments are made in unbuffered solutions (i.e. in solutions containing small amounts of strong acids) the result shows that a certain amount of a neutral salt must be present to depress the anomalies caused by an unequality in  $p_H$ .

This amount of salt is also generally sufficient to make the difference in conductivity between the two solutions negligible (see the experiments p. 76—77 below).

Unbuffered solutions for electrophoresis experiments can not be prepared as simply as buffered solutions, since the total amount of strong acid (free and bound) generally is quite different in the colloid and the supernatant liquid. The most direct way of preparing the solutions is to dialyze the mixture of protein and acid, a sufficient and equal quantity of salt being present from the beginning in both solutions. One may also, of course, after measuring the  $p_H$  of the protein-acid-salt-mixture, prepare a solution of equal  $p_H$  and salt content to be used as supernatant liquid, if the activity coefficient of the acid in the salt has been determined.

Some determinations according to the first mentioned procedure were made with phycoerythrin and phycocyan in fairly acid mixtures of HCl and KCl (see p. 76—77).

Experiments in unbuffered solutions of  $p_H$  values higher than 4.0—4.5 were made difficult by troubles in the  $p_H$ -measurements. It proved impossible to get reproducible potentials in HCl—KCl mixtures of these low acidities, even if only boiled out vessels and water free of carbon dioxide was used. At these  $p_H$  values, however, it is of importance to be able to make sure that the equilibrium has been reached, since the dialysis evidently proceeds more slowly.

Electrophoresis experiments with solutions that had been allowed to dialyze for about one week generally showed boundary anomalies, especially in the neighbourhood of the isoelectric points, where long times of experiment had to be used. Possibly the acidity changes produced by migration of hydrogen ions might be responsible for these effects. The risk of errors of this kind is of course great in unbuffered solutions (see p. 43).

To be able to perform measurements at  $p_H$  values above 4.5—5.0 it was found necessary to use a somewhat modified procedure. The difficulties in  $p_H$ -measurements were generally not experienced with solutions containing protein. It was assumed that this depended upon the buffering action of the protein itself. This suggested the following method: A solution containing protein of concentration 0.1 % was used as supernatant liquid. The protein concentration of the other solution was increased from 0.2 % to 0.3 %. In this case no marked boundary anomalies were observed and  $p_H$ -measurements could be performed.

On p. 76 will be described some experiments according to this method. In this



case the time of exposure when photographing the boundaries has to be increased 3—4 times.

It has been emphasized in the foregoing that the depression of the boundary anomalies is a necessary condition not only for obtaining the mobility of the colloid from the rate of migration of the boundaries, but also for the purpose of studying the uniformity of migration. In cases when the latter point can be considered less important the mobility may still be obtained in cases of boundary anomalies by a somewhat more general method of calculation. The method is, however, rather laborious and has only been used in a few cases. The principle of the method is, however, of a certain value for the estimation of errors in electrophoresis measurements caused by diffusion, as will be shown in the following.

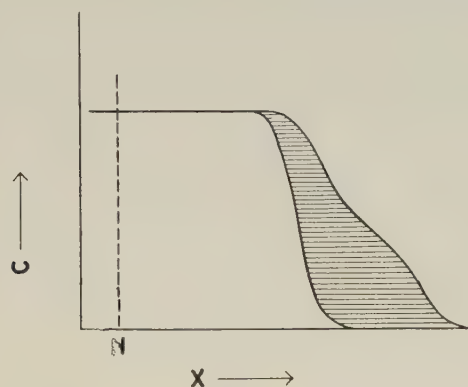


Fig. 12.

Fig. 12 shows two concentration-distance curves in a case of anomalous migration. At a level  $N$  at a sufficient distance from the boundaries the concentration of migrating substance remains unchanged, and also the potential gradient and the ionic medium. Therefore, in the time  $t$  the quantity  $\Delta m$  of substance migrating through  $N$  is given by

$$\Delta m = F u t q c, \quad (28)$$

where  $F$  is the potential gradient at  $N$ ,  $u$  the mobility,  $q$  the cross section area of the tube and  $c$  the concentration. As long as the concentration changes occurring at the very boundary do not reach the level  $N$ , the total quantity  $m$  of substance above  $N$  is independent of what happens at the boundary. This quantity  $m$  is obtained from the diagram by integration.

$$m = q \int_N^{\infty} c \, dx. \quad (29)$$

Therefore

$$\Delta m = m'' - m' = q \int_N^{\infty} (c'' - c') dx, \quad (30)$$

where the indices correspond to the two curves in the diagram. This integral is the lined area in fig. 12 and can be obtained by graphical integration. Then  $u$  is obtained from

$$u = \frac{\int_N^{\infty} (c'' - c') dx}{F t c}. \quad (31)$$

In this way we obtain the mobility at  $N$ , in the undisturbed part of the solu-

tion. It is seen that  $\frac{\int_N^{\infty} (c'' - c') dx}{c}$  is the migration distance of the substance in the time  $t$ .

Evidently this mode of calculation is in analogy to the transference method, especially in the form of ENGEL and PAULI (17).

An attempt was made to apply this method in the experiment with 0.35 % egg albumin in 0.02 n potassium chloride, mentioned above.

Table V.

0.35 % egg albumin in 0.02 n potassium chloride.  $p_H = 4.81$ . Conductivity of protein solution  $\kappa = 2.47 \times 10^{-3}$ , of supernatant liquid  $2.49 \times 10^{-3}$ . Migration anodic.

| $i \times 10^3$ | $t$ | $\Delta_L$ | $\Delta_R$ | $\Delta_M$ | $u_{20} \times 10^5$ |
|-----------------|-----|------------|------------|------------|----------------------|
| 2.209           | 60  | + 0.60     | - 0.60     | 0.60       | 1.42                 |
| 2.218           | 60  | (+ 1.52)   | - 0.60     | —          | 1.42                 |
| 2.217           | 120 | (- 2.27)   | + 1.18     | —          | 1.39                 |
|                 |     |            |            |            | 1.41                 |

(The meaning of the symbols in the above table see below p. 54.)

It is seen from the curves that direct measurement of the displacement is not possible, and that integration can be done only for the first time intervals, since afterwards the concentration at the bottom index changes. The integration was performed on millimeter paper, after the curves had been transformed into concen-

tration curves, and by dividing the areas  $\int (c'' - c') dx$  by the height  $c$  of the curve the migration distances in table V were obtained, as described above.

For the calculation of the mobility the values  $A_R$  and only the first value  $A_L$  were used.

For higher mobilities the application of the method is probably easier, since much shorter time of experiment can be used, and consequently considerable migration allowed to take place, before the disturbances reach the lower index.

Even in cases when we are sure that the conditions are constant at the level N, there is an important reservation to be made in this method of calculation (and similar methods to overcome the boundary anomaly difficulties, for example that of ENGEL and PAULI). The assumption was that substance was transported through the level N by electrophoretic migration only, and this is really the case if the migration is convection-free. But in the case of an inhomogeneous way of current, convections may arise on account of the migration. Let us again consider fig. 9.

A necessary condition of hydrostatic equilibrium is that  $\frac{\partial c}{\partial x} \leq 0$ , a condition that is of course not disturbed by the migration according to eq. (8) in an homogeneous way of current. Let us, however, assume that there exists a diffusion gradient of electrolytes across the boundary reaching into the region of constant colloid concentration  $c$  (as may often be assumed to be the case if the two media are different), causing a varying potential gradient or mobility. In the region of constant  $c$  ( $\frac{\partial c}{\partial x} = 0$ ) we obtain from eq. (15)

$$\frac{\partial c}{\partial t} = -uc \frac{\partial F}{\partial x} - Fc \frac{\partial u}{\partial x}. \quad (32)$$

If  $u$  and  $\frac{\partial F}{\partial x}$  or  $F$  and  $\frac{\partial u}{\partial x}$  have opposite signs (that is, if the migration will produce sharpening of the boundary, see p. 28 above)  $\frac{\partial c}{\partial t}$  must consequently be positive.

Therefore, at a certain place below the boundary, the concentration after some time should become greater than  $c$ , that is  $\frac{\partial c}{\partial x} > 0$ . The equilibrium is disturbed and convections must arise, that transport a certain amount of the substance downwards. In such cases even the method of determining the mobility by measuring the change of the amount of substance above a certain level in the tube is not correct. The direction of migration must be chosen so that no sharpening of the boundary to be observed occurs.

The result can also be expressed in the following way. If a sharp boundary migrates downwards in a tube with a rate of movement  $u \times F^1$ , where  $F^1$  is the



potential gradient at the very boundary, and if in the lower parts of the tube at a certain level  $N$  the potential gradient is equal to  $F$ , and  $F^1 > F$ , the amount  $u \times (F^1 - F) \times t \times q \times c$  is transported through  $N$  by convection and  $u \times F \times t \times q \times c$  by migration. ( $q$  is the cross section area of the tube.) The total amount, measured in the transference method, is therefore  $u F^1 t$  and not  $u F t$ .

### The diffusion.

According to eq. (10) and (11) for the migration in a homogeneous medium, the diffusion at the boundary should follow the same laws as are valid for the boundary at rest. If an originally sharp boundary is blurred by diffusion and if the change with time in concentration distribution at the boundary is followed for example with a photographic-microphotometric method similar to that used in this work, the diffusion constant can be determined (50). The following equation can be used for the calculation

$$D = C \times \frac{z^2}{t} \quad (33)$$

where  $z$  is half the distance between two diffusion column levels with concentrations  $3/4$  resp.  $1/4$  of the original,  $t$  the time of diffusion and  $C$  a constant equal to 1.099.

For the boundaries studied the time corresponding to the absolutely sharp boundary ( $z = 0$ ) is not known and therefore the equation was used in the form

$$D = C \times \frac{z^2 - z_0^2}{t - t_0} \quad (34)$$

where  $z_0$  corresponds to the first exposure. Now it should be observed that eq. (33, 34) are valid only if the boundary is absolutely sharp from the beginning or at least has been developed from an originally absolutely sharp boundary by diffusion only. In the experiments described in this work the boundary formation process probably acted disturbing (see above p. 20), and often the concentration distribution at the start was not even symmetrical with respect to the  $c/2$  level, as it should be for an ideal diffusion boundary. Moreover, the boundary anomalies are never quite eliminated. The application of eq. (34) to our case is therefore not quite justified.

In cases when a calculation of  $D$  from the curves has been tried strongly varying diffusion constants have been obtained, only of about the right order of magnitude. It is evident that changes in concentration distribution of the same order of magnitude as the diffusion cannot be discussed quantitatively in this work.

### The method of calculation.

In all determinations to be described in the following the migration velocity of the  $c/2$  level has been assumed equal to that of the migrating substance. This would be quite correct if no boundary anomalies occurred and if the boundaries were blurred by ideal diffusion only, since according to eq. (34) above the diffusion is symmetrical with respect to the  $c/2$  level. However, since it was found that the change in concentration distribution with time did not follow the diffusion equation exactly, a certain error may be introduced by this method of calculation.

To eliminate the effect of diffusion anomalies upon the mobility determinations the integration method of calculation was originally tried. However, it was found that the corrections obtained were small in most cases, and even when they were

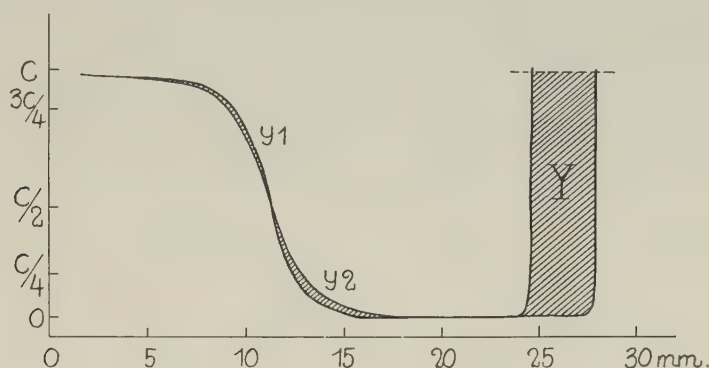


Fig. 13.

of considerable magnitude, the agreement between different values in the same experiment was not improved by this calculation method. It was concluded that the corrections obtained were of the same order of size as the other experimental errors.

The effect of the diffusion is not very marked in the experiments with hemocyanin, phycoerythrin, and phycocyan, which all have low diffusion constants compared to the other proteins investigated, egg albumin, serum albumin, and Bence Jones protein, which show greater blurring of the boundaries with time. (See the diagrams from the experiments below.) The effect is generally largest, for a certain time interval, in the beginning of an experiment.

To obtain an idea of the magnitude of the correction (in an unfavourable case) we may consider the experiment 4 with egg albumin, described below p. 55, the two first exposures in the left tube (curves 1 and 2 in lower image fig. 17). The calculation of the corrections was generally made with a somewhat simplified method of integration (fig. 13).

The two different curves are placed one above the other, so that the  $c/2$  points

coincide. The area to be integrated for obtaining the mobility is evidently equal to the area  $Y + y_1 - y_2$ . Therefore, according to eq. (31) we get

$$u = \frac{Y + y_1 - y_2}{F t c}. \quad (35)$$

But  $Y/c$  is evidently the distance of migration for the point  $c/2$ . If this distance is called  $\mathcal{A}$  we get

$$u = \frac{1}{F t} \times \left( \mathcal{A} + \frac{y_1 - y_2}{c} \right). \quad (36)$$

Therefore the migration of the level  $c/2$  gives correct values for the mobility if a correction is added which corresponds to the difference in shape between the curves and is calculated by integration.

To perform this integration exactly the curves should be drawn in concentration scale but as it is only a question of calculating a correction we may simply count the number of square millimeters for each concentration interval ( $c - 3c/4$ , and so on), and reduce the area thus obtained to correct concentration scale for each such interval separately. In the case described the areas are  $y_1 = 0.36c + 0.19c = 0.55c$ .  $y_2 = 0.31c + 0.90c = 1.21c$ .  $y_1 - y_2 = -0.66c$ .  $Y = 17.0c$ . The enlargement is  $5\times$ . The correction is therefore 0.13 mm whereas  $\mathcal{A}$  is 3.40 mm.

In most cases, however, the correction is smaller, the curves of the diagrams being almost congruent. Partly, the areas  $y_1$  and  $y_2$  are small as compared to  $Y$ , partly they are generally almost equal in size.

Cases when the boundaries are changed so strongly that the corrections become of considerable importance are observed with mixtures and non-uniform proteins. In such cases, however, a calculation of the average mobility by integration is of no interest.

We will now discuss some other sources of error and some factors that may be assumed able to influence the results.

### The heat convection currents.

The heat  $w$  watt produced per  $\text{cm}^3$  and second in a tube of cross section area  $q$   $\text{cm}^2$  if the current intensity is  $i$  ampere and the conductivity  $\kappa$  is obtained by

$$w = \frac{i^2}{q^2 \kappa}. \quad (37)$$

It is more convenient for the discussion to express  $w$  in terms of the potential gradient  $F$  volt/cm, instead of  $i$ , since for the comparison of the efficiency of



different arrangements for an electrophoresis experiment the potential gradients are essential. We get

$$w = F^2 \kappa, \quad (38)$$

therefore independent of the dimensions of the tube.

The rise in temperature in the tube for a given heat effect  $w$  can be reduced by good stirring of the thermostate and also by making the tube as narrow as possible since then its surface per  $\text{cm}^3$  becomes greater. As mentioned above, however, it is not possible to reduce the diameter of the tube without influencing other necessary conditions of experiment (the difficulties in obtaining sharp boundaries in narrow tubes, p. 19, the greater risk of boundary anomalies in solutions of the higher protein concentrations necessary in a narrow tube to give the right contrast, p. 19).

Some experiments were made for the special purpose of studying the effects of heat convection currents and the magnitude of the errors produced.

To obtain the effects as pure as possible experiments with alternating current were made with successively increasing potentials on the apparatus.

In table VI are shown the results of such an experiment with 0.2% phycocyan in m/25 potassium chloride solution.

Table VI.

| Exposure no. | Voltage $V$<br>volts | Time inter-<br>val $t$ minutes | Heat effect<br>per $\text{cm}^3$ $w$<br>watt | Distance of<br>$c/2$ from<br>upper index<br>mm |
|--------------|----------------------|--------------------------------|----------------------------------------------|------------------------------------------------|
| 1            | —                    | —                              | —                                            | 17.12                                          |
| 2            | 0                    | 60                             | 0                                            | 17.20                                          |
| 3            | 30                   | 60                             | $2.12 \times 10^{-3}$                        | 17.12                                          |
| 4            | 50                   | 60                             | $5.87 \times 10^{-3}$                        | 17.12                                          |
| 5            | 75                   | 60                             | $13.2 \times 10^{-3}$                        | 17.06                                          |
| 6            | 100                  | 30                             | $23.5 \times 10^{-3}$                        | 17.00                                          |
| 7            | 200                  | 20                             | $94.0 \times 10^{-3}$                        | 15.80                                          |

The voltage was measured with an electrodynamic voltmeter.

The conductivity was  $\kappa = 4.81 \times 10^{-3}$ .

To calculate the heat effect  $w$  from the voltage on the apparatus  $V$  one must know the potential gradient  $F$ . The ratio  $F/V$  is an apparatus constant independent of the solution used. It was calculated from the following data: In an experiment with a solution of conductivity  $1.437 \times 10^{-3}$  a current intensity of  $1.245 \times 10^{-3}$

amp. was obtained, when  $V$  was 51.5 volts. Therefore  $F/V = \frac{1.245 \times 10^{-3}}{51.5 \times q \times \kappa} = 0.0221$ .

Consequently  $w = (0.0221 V)^2 \times 4.81 \times 10^{-3}$ .

A marked change was first observed since the voltage had been increased to 75 volts: the boundary was sharpened and in its central part a fine column of upwards streaming dilute protein solution could be seen. It was hardly visible in transmitted light but could very well be observed by its strong fluorescence when a nickel-glass filter was put in. The effects became very marked when the voltage was increased. Fig. 14 shows a photograph of the first and the last exposure.

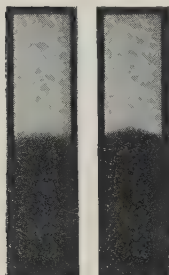


Fig. 14.

The photometric records are all almost completely congruent, except no. 7. In the table is given the distances of the levels  $c/2$  from the upper index, measured as usual from the curves.

From these results it is evident that in experiments with solutions of 0.2% protein concentration, there is a risk that heat convection currents may interfere, if effects greater than about  $10 \times 10^{-3}$  watt per  $\text{cm}^3$  are used.

In acetate buffers of conductivity  $\kappa = 1.44 \times 10^{-3}$  current intensities of  $1.2$ — $1.6 \times 10^{-3}$  amp. were generally used, which corresponds to a  $w$  value of  $1.7$ — $3.1 \times 10^{-3}$  watt/ $\text{cm}^3$ . In phosphate buffers of  $\kappa = 1.24 \times 10^{-3}$  currents of  $1$ — $2 \times 10^{-3}$  amp. were used, corresponding to  $w$  values of  $1.4$ — $5.6 \times 10^{-3}$  watt/ $\text{cm}^3$ . In other cases also care was taken not to exceed the maximum allowable effect thus determined.

#### The influence of migration of electrolytes from the electrode vessels and of the electrode reactions.

It has already been mentioned that reversible silver-silver chloride electrodes were used in a saturated potassium chloride solution which filled the lower parts of the electrode tubes, and that reversible electrodes were found quite indispensable in this work, where especially small mobilities had to be measured.

In this arrangement care has to be taken that the chloride does not reach the boundaries in an experiment by electrical migration or by diffusion. That this was not the case in the experiments to be described is evident from the constancy of the current intensity and may also be seen from the following discussion.

The cross section area of the electrode vessels was  $10.7 \text{ cm}^2$ . In a solution of conductivity  $1.44 \times 10^{-3}$  and with a current of  $1.5 \times 10^{-3}$  ampere (as in most experiments in 0.02 n acetate buffers), the potential gradient is therefore  $\frac{1.5 \times 10^{-3}}{10.7 \times 1.44 \times 10^{-3}}$  — approximately equal to 0.1 volt/cm; in the layers containing potassium chloride it is still lower. Since the mobility of K and Cl ions is  $65 \times 10^{-5} \text{ cm}^2 \text{ sec}^{-1} \text{ volt}^{-1}$  these ions will migrate 0.234 cm. per hour. In each tube the KCl solution reaches about 2 cm. above the bottom and the distance from the bottom to the side tube is 10 cm. Since the experiments at this current intensity seldom lasted more than

6—8 hours, it is evident that there is no danger of any potassium chloride reaching the boundaries in this way.

The diffusion is probably more dangerous. However, it was found by a special diffusion experiment, in which the apparatus was filled in the ordinary way, and samples were carefully drawn out with a fine pipett from a level 10 cm. above the bottom of the electrode tube, that chloride ions do not reach this level in a sufficient amount to give opalescence with  $\text{AgNO}_3$  until after 20 hours.

Of the ions present the hydrogen ions are of special importance on account of their great influence on protein mobility and their exceptionally high mobility and diffusion constant (which are about 5 times as great as for potassium and chlorine ions). In buffered solutions there is probably no risk of concentration changes of hydrogen ions. In unbuffered solutions (mixtures of  $\text{HCl}$  and  $\text{KCl}$ ) some anomalies were found that may be explained as the result of such changes (see p. 34 above).

The volume changes in the electrode vessels produced by the reactions occurring at the electrodes may give rise to a movement of the solution as a whole through the tube, and therefore possibly influence the mobilities observed. A discussion of this source of error in measurements of mobilities was given by G. N. LEWIS (51). The effect is of importance if the quantities of electricity to be used are great.

It was thought necessary to discuss this effect together with the other sources of error, since the mobilities to be measured are small, and since one important result of the measurements, the position of the isoelectric point, might also be influenced. However, it was found that the effect in our case is insignificant. This can be shown in the following way.

We consider the change in volume taking place in one electrode tube when one faraday is sent through the apparatus. This change may be due to two factors: the migration of substance into the electrode tube and the electrode reaction.

The transference number of acetate ion in sodium acetate is 0.44 (calculated from the mobilities in LANDOLT-BÖRNSTEIN 5. Aufl., Erg.-Bd. I, p. 620).

At the anode, therefore, the following changes have taken place:

Increase: 0.44 mol acetate ions, 1 mol  $\text{AgCl}$ .

Decrease: 0.56 mol sodium ions, 1 mol chlorine ions and 1 mol  $\text{Ag}$ .

Likewise at the cathode:

Increase: 0.56 mol sodium ions, 1 mol chlorine ions and 1 mol  $\text{Ag}$ .

Decrease: 0.44 mol acetate ions, 1 mol  $\text{AgCl}$ .

If the total volume changes are denoted  $\Delta v_A$  and  $\Delta v_K$ , and the specific molar volumes  $v$ , we get

$$\Delta v_A = -\Delta v_K = 0.44 v_{Ac} + v_{AgCl} - 0.56 v_{Na} - v_{Cl} - v_{Ag}. \quad (39)$$

If the specific molar volumes of the ions in  $\text{NaCl}$  and  $\text{NaAc}$  are supposed to be additive properties we get, by adding  $(0.44 v_{Na} - 0.44 v_{Na})$



$$\Delta v_A = -\Delta v_K = 0.44 v_{NaAc} + v_{AgCl} - v_{NaCl} - v_{Ag}. \quad (40)$$

According to the densities given in LANDOLT-BÖRNSTEIN'S Tables I, p. 425 the specific molar volumes of sodium chloride and sodium acetate in solution are approximately the same and equal to 17 cm<sup>3</sup>. The specific weight of AgCl is 5.37 (L. B. I, p. 307) and of Ag 10.5 (L. B. I, p. 290). Therefore

$$\Delta v_A = -\Delta v_K = 5.9 \quad (41)$$

Consequently, a certain level in the solution is moved over a volume of 5.9 cm<sup>3</sup> when one faraday is passed through. This corresponds to a migrated distance of  $\frac{5.9}{q}$  cm. The mobility  $u$  is therefore

$$u = \frac{5.9}{q \times F \times t} = \frac{5.9 \times z}{i \times t} \quad (42)$$

according to eq. (3). Since  $i \times t = 96500$ ,  $z = 1.44 \times 10^{-3}$  we get  $u = 8.8 \times 10^{-8}$  cm<sup>2</sup> sec<sup>-1</sup> volt<sup>-1</sup>.

The errors of experiment are in the most accurate measurements about  $2 \times 10^{-6}$  cm<sup>2</sup> sec<sup>-1</sup> volt<sup>-1</sup>. The effect discussed can therefore be entirely neglected.

There is, however, another cause of movement of the solution as a whole: the displacement of the heavier layer of protein in the U-tube causes a hydrostatic pressure that must be counterbalanced by a displacement of the solution as a whole. In consequence, at equilibrium the surface levels in the electrode tubes are not exactly at the same height. If the boundary has migrated the distance  $l$ , if the densities of protein and supernatant solutions are  $\rho_p$  and  $\rho$  and the displacement of the surface in one electrode tube is  $h$  we get (see fig. 15)

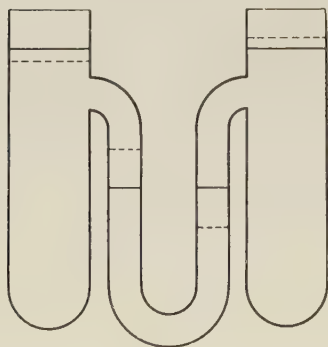


Fig. 15.

$$h = \frac{\rho_p - \rho}{\rho} \times l \quad (43)$$

This corresponds to a movement of the solution in the U-tube over a distance  $\Delta l$ :

$$\Delta l = \frac{S}{q} \times \frac{\rho_p - \rho}{\rho} \times l \quad (44)$$

Here  $S$  is the free surface of the solution in one electrode tube, and  $q$  the

cross section area of the U-tube. The true displacement of the boundary is therefore  $l + \Delta l$ . We get approximately

$$\frac{\Delta l}{l} = \frac{S}{q} (q_p - q) \quad (45)$$

In the apparatus used  $S = 1.77 \text{ cm}^2$ ,  $q = 0.7615 \text{ cm}^2$ .

For a 1% protein solution  $q_p - q$  is about 0.0025. This gives a correction of 0.6% in  $l$ . Generally more dilute solutions are used, and the error becomes still smaller.

It should be observed, however, that if the free surfaces in the electrode tubes are large, as in the SVEDBERG-TISELIUS apparatus (27) where  $S = 12.6 \text{ cm}^2$ , the correction may become of importance. For the apparatus mentioned, with 1% solutions, it amounts to 3%.

### The endosmosis.

The endosmosis at the walls of the apparatus can not, in tubes as wide as those used in this work, give rise to any transport of the solution as a whole. However, it may contribute to the blurring of the boundaries. Any definite evidence of endosmotic effects could not be obtained.

### Optical and photographic sources of error.

A discussion of optical and photographic sources of error in a work of similar kind has been given by RINDE in his work with the ultracentrifuge (52). I will therefore only mention here some circumstances of importance in this particular method.

As was mentioned above, the U-tube had been made of fused quartz, and it was not quite free from small bubbles and similar imperfections that became specially disturbing in photographs of low contrast. The microphotometric records are therefore, as a rule, more irregular than those obtained in an apparatus with walls of a more perfect material. An attempt was made to construct an apparatus of ebonite with plane crystalline quartz plates and it was used in some experiments. However, it proved to be much more difficult to avoid heat convection currents in this apparatus and therefore it was not used any more. It is probably important that the solution in the U-tube is cooled symmetrically, and therefore the cylindrical tube should be the best.

As a consequence of the cylindrical shape of the tube the blackening on the plate at a certain level in the protein region is not even but is smallest in center and increases, first slowly and then very rapidly towards both sides. In the

microphotometer, the registering was always made with the light spot in the middle of the image. It was found that an error of 0.5 mm. in this adjustment did not change the record obtained markedly. (The light strip had a length of 1 mm.)

Errors often occur in the blackening of the images owing to fog and similar plate imperfections, and form a serious source of error. Many exposures, sometimes whole experiments, had to be discarded on account of the constant concentration parts of the diagrams not coinciding.

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### The preparation of the proteins.

The following proteins have been studied in this work: Egg albumin, serum albumin, phycoerythrin from *Ceramium rubrum*, phycocyan from *Aphanizomenon flos aquae*, hemocyanin from *Helix pomatia*, Bence Jones protein and serum globulin.

It was thought important to make the choice among proteins that are obtainable in crystalline form. This is possible for all the substances mentioned, except Bence Jones protein and serum globulin. It is also of great value, that all these proteins have been studied in the ultracentrifuge and were found to be, within certain  $p_H$ -limits, stable and uniform as regards molecular weight.

It was further necessary to choose proteins of sufficient solubility in the neighbourhood of the isoelectric point, since the electrophoresis in this region was the main purpose of study.

*Egg albumin.* Egg albumin together with ovoglobulin and ovomucoid is found in egg white. It was first prepared in crystalline state by HOFMEISTER (53). The method was modified by HOPKINS and PINKUS (54) and by SØRENSEN (55).

According to SØRENSEN the globulin is precipitated by half saturation of the egg-white with ammonium sulphate and the precipitate filtered off. The filtrate is then brought to crystallization by addition of still more ammonium sulphate and some sulphuric acid, to give it the optimal  $p_H$  for crystallization.

This method was followed with some changes. Instead of the very tedious filtering described by SØRENSEN a centrifuge was used both for separating off the globulin precipitate and the egg albumin crystals, as well as for washing these crystals with ammonium sulphate solution. After two or three recrystallizations the crystals were dissolved and dialyzed in the cold against distilled water in collodion bags for about one week. The solution and the water were kept saturated with toluene as a disinfectant. The dialyzed solution had a conductivity of  $2-3 \times 10^{-4}$ . It was then electrodialyzed in a Pauli electrodialyser with parchment membranes for 48 hours, during the last 24 hours with a voltage of 220 volts direct on the apparatus. The conductivity then dropped down to  $1-2 \times 10^{-5}$ . The concentration of solution was determined by drying a sample of one cm<sup>3</sup> at 105°. The solution was kept in an ice-box, with toluene as a preservative.

Egg albumin absorbs in the short-wave ultraviolet region only (see p. 12).

*R-Phycoerythrin.* R-Phycoerythrin and the closely related R-phyococyan are well-defined, easily crystallizable proteins that are found together in certain algae. The letter R is used to distinguish the substances that are found in algae of the *Rhodophyceae* group from those found in the *Cyanophyceae* group, which are denoted with a C. (See SVEDBERG and KATSURAI (56)). These proteins have been known since long ago; they were first mentioned by KÜTZING in 1843 (57). They were obtained in pure state by KYLIN (58), who described the preparation from *Ceramium rubrum* and investigated their chemical properties, elementary composition and light absorption in the visible.

Recently LEMBERG (59) has published a thorough investigation of the properties of these substances. In this work are found literature references also to recent papers on the subject.

Phycoerythrin has a strong red colour, with yellow fluorescence, phyococyan is blue, with red fluorescence. One would expect that in analogy to hemoglobin and hemocyanin the colour would be due to the presence of heavy metals. However, according to KYLIN and to LEMBERG, this does not seem to be the case.

For preparation, KYLIN's procedure was followed. About 20 kilograms (wet) of *Ceramium rubrum* were collected at Kristineberg's Zoological Station, Fiskebäckskil, where the extraction and first preparation were also carried out. The material was extracted three times with rain-water, each time in a quantity just sufficient to cover the material in the vessel used. The extract was filtered through filter cloth and the material was pressed out as much as possible. It was precipitated completely by addition of 25 % of its weight of ammonium sulphate. The precipitate was filtered and filled on bottles for transport.

This precipitate consisted mainly of slimy carbohydrates but contained the whole quantity of phycoerythrin and phyococyan present. The further purification was done in Upsala. The precipitate (weight 2 kilograms) was extracted by means of distilled water and precipitated with increasing amounts of ammonium sulphate up to 20 % of the weight of the solution. The first fractions obtained contained the main part of the phycoerythrin and these were further purified by 4 recrystallizations. For the separation of the phyococyan from phycoerythrin use was also made of their different rates of solubility, as described by KYLIN.

The last precipitate of phycoerythrin was dissolved in as small as possible a quantity of distilled water and was dialyzed against distilled water in the cold, until it started to precipitate. As being more globulin-like than egg albumin, R-phycoerythrin and R-phyococyan require a certain amount of salt to keep them in solution. This quantity is, however, probably very small; the precipitation does not set in until the conductivity has fallen below  $10^{-4}$ . The precipitate was redissolved in sodium acetate so that a solution of 0.96 % phycoerythrin in 0.002 N acetate was obtained. A stock solution containing 0.01 M. phosphate buffer (1:1) was also prepared. Purification by electrodialysis cannot be carried out, since part of the phycoerythrin becomes insoluble.

The amount of R-phycocyan obtained was insufficient for electrophoresis experiments.

Phycocerythrin has strong light absorption in the visible as well as in the long-wave and the short-wave ultraviolet. (56, 60.)

*C-Phycocyan.* This protein was prepared from an alga of the *Cyanophyceae* group: *Aphanizomenon flos aquae*. Since its preparation in pure state from this source has not been described before and since by the procedure large quantities of this well-defined protein can easily be obtained, a more detailed description of the extraction and purification may perhaps be of interest.

*Aphanizomenon flos aquae* is a green or bluish-green alga that is found floating on the surface of lakes. BORESCH (61) studied the blue-coloured extract from this plant spectroscopically.

The material was collected from the surface of a creek of lake Walloxen, near Upsala, with flat landing nets of brass wire gauze. It was strained off on filter cloth and a mass of spinach-like consistency was obtained. About 30 litres were collected. After standing for one night at room temperature, autolysis set in, gas evolution could be noticed and the mass was less thick in consistency. A smell similar to that of butyric acid could be perceived. Toluene was added after 3 days in a rather large quantity, since it was apparently absorbed to some extent by the material. After two days more the extract was centrifuged for about one hour at 2000 r.p.m. About half of the volume of the extract could then be pipetted off. It showed a very strong greenish-blue colour and red fluorescence. The residue was discarded, even though it would probably have been possible to gain some more substance from this. The solution obtained was filtered and yielded a yellowish-brown, slimy residue on the filters.

The total amount of solution was precipitated by the addition of 7 volumes of concentrated ammonium sulphate solution (600 gr./litre) to 10 volumes of filtrate. In this way a large amount of blue precipitate was obtained, that could easily be centrifuged off from the green mother liquor. The precipitate dissolved very easily in distilled water. This solution was filtered.

An attempt was then made to bring the filtrate thus obtained to crystallization by addition of gradually increasing amounts of ammonium sulphate. The solution started to precipitate when about  $\frac{1}{6}$  of the volume of saturated ammonium sulphate solution was added, but even 0.4 of the volume of ammonium sulphate was not enough to complete the precipitation, and the mother liquor was still blue or bluish-green. The precipitates were investigated under the microscope, but no crystals could be seen. The next step was to add the sulphate gradually with time intervals of one day, each time in a volume equal to one tenth of the original, but the precipitates obtained still did not contain any crystals, as far as could be seen under the microscope.

The solution was therefore simply reprecipitated 3 times by addition of 4 volumes



of sulphate to 10 volumes of solution. The mother liquor in the two last precipitations had a pure blue colour, and the smell of butyric acid had disappeared. Some water was added to the last precipitate and it was kept with toluene at  $0^{\circ}$ .

After about 2 weeks small glittering crystals were observed on the walls of the bottle, and microscopical investigation showed that the precipitate had turned almost completely crystalline. A photograph of such crystals is found in the work of SVEDBERG and KATSURAI (56). The crystals were exceptionally big for being of a protein (length more than 0.1 mm.).

The crystals were centrifuged off and dissolved in an amount of water as small as possible. When placed in the ice-box this solution crystallized again, and in this way some of the material was recrystallized twice.

Stock solutions containing 0.001 N acetate (1:1) and phosphate (1:1) buffers were prepared. The protein concentration was 1.10 %.

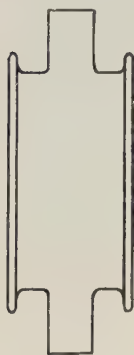


Fig. 16.

Electrophoresis experiments showed that the mother liquor especially and also the crystals from the first crystallization contained a foreign substance with light absorption in the long-wave and in the short-wave ultraviolet regions, but not in the visible. These experiments will be described in the following. The impurity was not found in the recrystallized material.

An attempt was made to purify the phycocyan from the impurity by another method: electrophoretic separation. For this purpose use was made of an electrophoresis phenomenon that is often observed in electrodialysis. It has been used earlier for the purification of starch by SAMEC and HAERDTL (62). In the electrodialysis of not too dilute colloid solutions in a horizontal PAULI electrolysers it is often found that after some time a sharp boundary is formed, separating the colloid from the supernatant liquid. This boundary gradually sinks down, and the sol becomes more concentrated. The phenomenon has been investigated especially by BLANK and VALKÓ (63). The explanation is that colloid is transported by electrophoresis towards one membrane. Therefore a layer of concentrated colloid is formed here, whereas at the other membrane a layer of colloid-free solution appears. On account of the gravity force, convections carry the two layers to the bottom and the top of the apparatus and this results in the formation of the horizontal boundary.

The purification of the phycocyan was performed in the following way. In an ordinary PAULI electrolysers the middle cell was replaced by a cell with two tubes according to fig. 16. A rubber stopper with a glass stopcock was placed in the lower tube. The cell was filled with the impure phycocyan solution (dialyzed material, conc. 1.50 %) to which ammonia was added to a concentration of 0.002 N. Parchment membranes were used. The outer liquid was 0.002 N ammonia. This solution was allowed to stream slowly through the apparatus from a bottle containing about 4 litres, first passing through one cell and then through the other, so

that the electrolysis products might mix with each other. The electrodes (of platinum) were kept at a distance of about 10 mm. from the membranes. A current density of 2 milliamps per square centimeter was sent through. After one hour most of the protein had already collected in the lower tube. It was tapped off and diluted with 0.002 n ammonia and the procedure repeated. After three experiments the concentrated protein was diluted with distilled water and put to dialysis. An electrophoresis experiment showed that it was free from the impurity: no trace of the second boundary formed in the experiments with the impure material could be observed.

Light absorption determinations on this protein are found in the work of SVEDBERG and KATSURAI (56). The absorption in the visible is different from that of R-phycocyan, whereas the long-wave and short-wave ultraviolet absorption curves are similar, at least in shape.

*Helix Hemocyanin.* Hemocyanins are found in the blood of certain lower animals: *Molusca*, *Crustacea*, and *Arachnoides* (64). Like hemoglobin in the blood of the higher animals they carry the respiratory function. They, however, contain copper instead of iron. The colour is blue in reflected light, and yellowish-red in transmitted light.

The preparation is very simple. According to DHÉRE (65) *Helix* hemocyanin can be crystallized by dialysis of the blood against distilled water and this method was used. The blood from thirty snails, *Helix pomatia*, was dialyzed in collodion bags at 0° for three weeks. The crystalline precipitate formed was dissolved in sodium acetate to a solution of 0.002 n acetate concentration. The protein concentration was 1.0 %. A small amount was also dissolved in 0.002 m sodium phosphate.

The light absorption was studied among others by SVEDBERG and CHIRNOAGA (66). The absorption in the visible and in the long-wave ultraviolet is much weaker than in the case of other coloured proteins.

*Serum albumin, serum globulin, Bence Jones protein.* These proteins were put at my disposal by the courtesy of Mr. B. SJÖGREN, Upsala, who had prepared these solutions for his investigations with the ultracentrifuge. A description of the methods of preparation used by him are found in (67), (68), and (69).

*Serum albumin* was obtained from horse serum and separated from the serum globulin by half saturation with ammonium sulphate. The globulin was precipitated and filtered off and the albumin was crystallized in the filtrate by addition of ammonium sulphate. The solution of crystals was dialyzed and electrodialed.

*Serum globulin.* The precipitate mentioned above was washed with half saturated ammonium sulphate solution and dissolved in 10 % sodium chloride solution. It was reprecipitated, washed and dissolved twice more. The final solution was

dialyzed against a phosphate buffer of  $p_H$  5.5, 0.19 m in  $KH_2PO_4$  and 0.009 m in  $Na_2HPO_4$ .

Both these proteins absorb only in the short-wave ultraviolet. Absorption measurements were made by SVEDBERG and SJÖGREN (67).

*Bence Jones protein.* This protein occurs in the human urine in the case of certain diseases, especially myelomas. (See WELLS, Chemical Pathology, p. 596, Saunders Co., Philadelphia and London, 5th ed., 1925.)

It was precipitated from the urine by addition of the double volume of saturated ammonium sulphate, and the material obtained was reprecipitated once. After dialysis for 6 days against pure water it was electro-dialyzed.

Light absorption measurements were made by SVEDBERG-SJÖGREN (68). The protein absorbs only in the short-wave region.



## Measurements of the electrophoretic mobilities of the proteins in electrolyte solutions of varying acidity.

### 1. Experiments in buffered solutions.

Except for serum globulin the isoelectric points of the proteins studied all fall within the  $p_H$ -region of the acetic acid — sodium acetate buffer. Most of the proteins soluble at their isoelectric points belong to this group.

As was to be expected the mobility does not depend upon  $p_H$  solely; other ions also interfere. Therefore the experiments in acetate buffers were made at constant sodium acetate concentration (generally 0.02 N). It is a great advantage of buffers of this type that they make a variation of  $p_H$  possible with only very small changes in the total ionic medium. For buffers like the mixture of  $\text{Na}_2\text{HPO}_4$  and  $\text{KH}_2\text{PO}_4$  this is not possible, a change in  $p_H$  is accompanied by a change in the composition of the ions present.

This question is of importance for all investigations made in buffer solutions. If thermodynamical properties are measured the application of the quantity »ionic strength» introduced by LEWIS and RANDALL (70) to determine the sum of the interionic effects in an electrolyte medium furnishes a way of choosing the composition of a buffer so that varying  $p_H$  and constant interionic effects are obtained. Tables for calculation of mixtures of this kind have been given by COHN (71).

When, however, non-thermodynamical properties have to be studied, it is not certain that this way is correct. The principle of the ionic strength is based upon activity measurements in dilute solutions and is also a consequence of the DEBYE-HÜCKEL theory of strong electrolytes. There are not yet sufficient observations of electrophoresis in different salt media to decide whether a similar rule is valid for the mobility also.

To follow some definite rule, however, the measurements in phosphate buffers were made at the same ionic strength as used in the acetate buffers (except in some experiments described in the last chapter, which were made for the main purpose of studying the uniformity of migration). Under the conditions mentioned the  $p_H$  and conductivity of the buffer and the protein-buffer mixture were almost always equal, within the experimental errors (0.01—0.02 in  $p_H$ ,  $^{1/2}$ —1 % in the con-

ductivity). In a few exceptional cases of poor buffering, the differences became greater. This is specially remarked below.

The conductivity values of the 0.02 n sodium acetate buffers are given in the following table VII.

Table VII.

Conductivities of sodium acetate buffers 0.02 n with respect to sodium acetate (20°.0).

| $p_H$   | $\kappa \times 10^8$ |
|---------|----------------------|
| 5.6—4.8 | 1.44                 |
| 4.8—4.2 | 1.45                 |
| 4.2—4.1 | 1.46                 |
| 4.1—4.0 | 1.47                 |
| 3.94    | 1.48                 |
| 3.67    | 1.49                 |
| 3.34    | 1.54                 |

In the following tables  $i$  is the mean current intensity in ampere for the time interval in question,  $t$  the time in minutes,  $\mathcal{A}_L$  and  $\mathcal{A}_R$  the distances of migration in mm,  $\mathcal{A}_M$  the mean value of  $\mathcal{A}_L$  and  $\mathcal{A}_R$ ,  $u$  the mobility in  $\text{cm}^2 \text{sec}^{-1} \text{volt}^{-1}$ . The + and — sign in front of  $\mathcal{A}_L$  and  $\mathcal{A}_R$  mean upwards resp. downwards movement.

All determinations were made at 20°.0.

From the diagrams obtained, when the mobility is plotted against  $p_H$ , the isoelectric points are obtained as the points of intersection between the curve and the axis of zero mobility. Since the mobility- $p_H$  curve mostly does not deviate much from a straight line, the slope of this curve together with the isoelectric point, can be said to characterize the function mentioned in each case. The value  $\frac{du}{dp_H}$  for the isoelectric point has therefore been calculated in every case.

### Experiments in sodium acetate-acetic acid buffers.

*Egg albumin.* All determinations were made with a protein concentration of 0.35 % (except no. 9). The exposures were taken in short-wave ultraviolet.

Table VIII.

Exp. 1. Sodium acetate 0.02 n. Acetic acid 0.048 n.  $p_H = 4.27$ . Migration cathodic.

| $i \times 10^3$ | $t$ | $\mathcal{A}_L$ | $\mathcal{A}_R$ | $\mathcal{A}_M$ | $u_{20} \times 10^5$ |
|-----------------|-----|-----------------|-----------------|-----------------|----------------------|
| 1.645           | 45  | —1.14           | +1.48           | 1.31            | 3.26                 |
| 1.646           | 124 | —3.82           | +3.36           | 3.34            | 3.01                 |
| 1.021           | 60  | +0.94           | —1.08           | 1.01            | <u>3.03</u>          |
|                 |     |                 |                 |                 | 3.1                  |

Table IX.

Exp. 2. Sodium acetate 0.02 n. Acetic acid 0.034 n.  $p_H = 4.44$ . Migration cathodic.

| $i \times 10^8$ | $t$ | $A_L$ | $A_R$ | $A_M$ | $u_{20} \times 10^5$ |
|-----------------|-----|-------|-------|-------|----------------------|
| 1.608           | 120 | -1.32 | +1.76 | 1.54  | 1.46                 |
| 1.615           | 60  | +0.70 | -0.92 | 0.81  | 1.53                 |
| 1.600           | 121 | +1.34 | -1.40 | 1.87  | <u>1.29</u>          |
|                 |     |       |       |       | 1.4                  |

Table X.

Exp. 3. Sodium acetate 0.02 n. Acetic acid 0.02 n.  $p_H = 4.68$ . Migration anodic.

| $i \times 10^8$ | $t$ | $A_L$ | $A_R$ | $A_M$ | $u_{20} \times 10^5$ |
|-----------------|-----|-------|-------|-------|----------------------|
| 1.608           | 120 | -1.48 | +1.32 | 1.40  | 1.33                 |
| 1.595           | 210 | +3.14 | -2.40 | 2.77  | 1.51                 |
| 1.625           | 185 | -2.24 | —     | 2.24  | 1.36                 |
| 0.300           | 821 | +2.02 | —     | 2.02  | <u>1.50</u>          |
|                 |     |       |       |       | 1.4                  |

Table XI.

Exp. 4. Sodium acetate 0.02 n. Acetic acid 0.012 n.  $p_H = 4.90$ . Migration anodic.

| $i \times 10^8$ | $t$ | $A_L$ | $A_R$ | $A_M$ | $u_{20} \times 10^5$ |
|-----------------|-----|-------|-------|-------|----------------------|
| 1.608           | 114 | +3.40 | -3.50 | 3.45  | 3.45                 |
| 0.955           | 75  | -1.36 | +1.34 | 1.35  | 3.45                 |
| 0.980           | 60  | -1.08 | +1.10 | 1.09  | 3.39                 |
| 1.605           | 86  | -2.32 | +2.76 | 2.54  | <u>3.36</u>          |
|                 |     |       |       |       | 3.4                  |

Table XII.

Exp. 5. Sodium acetate 0.02 n. Acetic acid 0.008 n.  $p_H = 5.04$ . Migration anodic.

| $i \times 10^8$ | $t$ | $A_L$ | $A_R$ | $A_M$ | $u_{20} \times 10^5$ |
|-----------------|-----|-------|-------|-------|----------------------|
| 1.593           | 60  | +2.34 | -2.70 | 2.52  | 4.82                 |
| 1.585           | 36  | -1.30 | +1.62 | 1.46  | 4.68                 |
| 0.920           | 75  | -2.02 | +1.78 | 1.90  | 5.03                 |
| 0.252           | 783 | -5.40 | +5.16 | 5.28  | <u>4.89</u>          |
|                 |     |       |       |       | 4.9                  |



Table XIII.

Exp. 6. Sodium acetate 0.02 n. Acetic acid 0.0045 n.  $p_H = 5.27$ . Migration anodic.

| $i \times 10^3$ | $t$ | $A_L$ | $A_R$ | $A_M$ | $u_{20} \times 10^5$ |
|-----------------|-----|-------|-------|-------|----------------------|
| 1.606           | 60  | +3.70 | -3.22 | 3.46  | 6.56                 |
| 1.602           | 65  | -3.88 | +3.72 | 3.80  | 6.67                 |
| 0.725           | 60  | +1.62 | -1.48 | 1.55  | 6.51                 |
| 0.247           | 750 | -6.76 | +6.80 | 6.78  | <u>6.69</u>          |
|                 |     |       |       |       | 6.6                  |

In fig. 17 are shown the curves drawn from the records in exp. 4. Fig. 18 gives a graphical representation of the results. The isoelectric point is evidently 4.55, and  $\frac{du}{dp_H} = 10.4 \times 10^{-5}$  in this point. In exp. 3 the two last exposures in the right tube were spoiled on account of a spot on the plate.

In some of these experiments the current intensity was varied, but not beyond the value where heat convection currents may appear (p. 40). The results prove

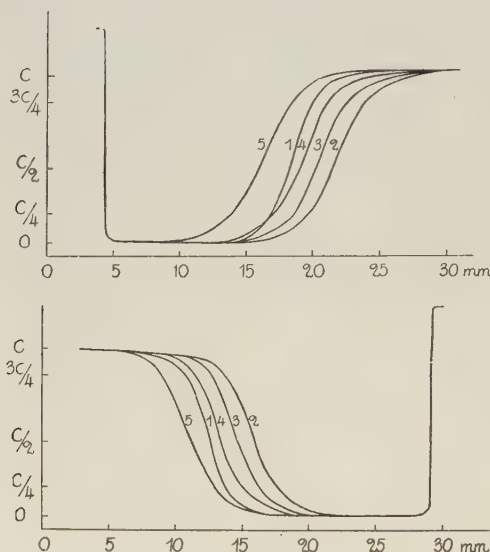


Fig. 17.

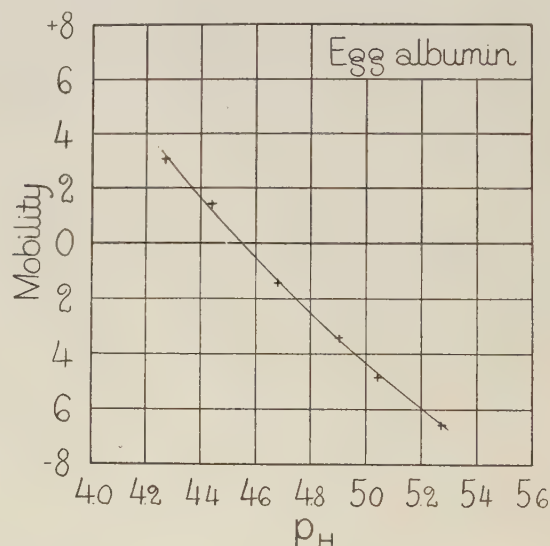


Fig. 18.

that the rate of movement is proportional to the potential gradient. Similar experiments were made in several other cases (see for example the experiments in phosphate buffer p. 71).

The foregoing experiments were made with a material that had been recrystallized three times and electro-dialyzed. To ascertain if the method of preparation influenced the electrophoresis, another sample of egg albumin was prepared (half a year later), by one crystallization only and dialysis for three weeks (no electro-dialysis). The following two determinations were made with this material (tables XIV and XV).

Table XIV.

Exp. 7. Sodium acetate 0.02 n. Acetic acid 0.017 n.  $p_H = 4.74$ . Migration anodic.

| $i \times 10^3$ | $t$ | $A_L$ | $A_R$ | $A_M$ | $u_{20} \times 10^5$ |
|-----------------|-----|-------|-------|-------|----------------------|
| 1.520           | 250 | +3.90 | -4.12 | 4.01  | 1.93                 |
| 1.520           | 105 | -1.56 | +1.64 | 1.60  | <u>1.83</u>          |
|                 |     |       |       |       | 1.9                  |

Table XV.

Exp. 8. Sodium acetate 0.02 n. Acetic acid 0.035 n.  $p_H = 4.43$ . Migration cathodic.

| $i \times 10^3$ | $t$ | $A_L$ | $A_R$ | $A_M$ | $u_{20} \times 10^5$ |
|-----------------|-----|-------|-------|-------|----------------------|
| 1.520           | 220 | -2.80 | +3.10 | 2.95  | 1.61                 |
| 1.520           | 120 | +1.80 | -1.80 | 1.80  | <u>1.80</u>          |
|                 |     |       |       |       | 1.7                  |

The migration was as uniform as in the measurements above.

From these two determinations we obtain the isoelectric point  $p_H^{\circ} = 4.58$  and  $\frac{du}{dp_H} = 11.6 \times 10^{-5}$ . There is therefore perhaps a small difference in properties between the two materials, but it is on the limit of the experimental errors.

In this connection it should be mentioned that in the preliminary work of SVEDBERG and the author on the electrophoresis of egg albumin in 0.02 n acetate buffer solutions a non-crystallized, dialyzed and electro-dialyzed material was used.

The isoelectric point was found at  $p_H$  about 4.65, and  $\frac{du}{dp_H} = 10.6 \times 10^{-5}$  (reduced to 20° by correcting for the change in viscosity, since the experiments were made at 13°.5). However, these experiments were not very accurate.

An experiment with dialyzed egg-white will be described in the last chapter (p. 94).

The following experiments were all made with the material used in exp. 1—6 above.

To ascertain if a change in protein concentration would influence the mobility markedly, the following experiment with 0.2 % protein concentration was made (hydroquinone developer was used to increase the contrasts). (Table XVI.)

Table XVI.

Exp. 9. Sodium acetate 0.02 n. Acetic acid 0.008 n.  $p_H = 5.05$ . Migration anodic.

| $i \times 10^3$ | $t$ | $A_L$ | $A_R$ | $A_M$ | $u_{20} \times 10^5$ |
|-----------------|-----|-------|-------|-------|----------------------|
| 1.603           | 60  | +2.52 | -2.40 | 2.46  | 4.67                 |
| 1.605           | 60  | +2.32 | -2.58 | 2.45  | 4.65                 |
| 1.598           | 60  | -2.38 | +2.60 | 2.49  | <u>4.74</u>          |
|                 |     |       |       |       | 4.7                  |

If the result is compared to the mobility obtained with 0.35 % protein concentration in exp. 5 just related, it is seen that, within the experimental errors, the influence of protein concentration on the mobility is negligible. This is also evident from the fact, that a marked concentration effect would have given rise to boundary anomalies, which has not been observed.

On the other hand, the buffer concentration has probably some influence on the mobility (see also p. 74). The concentration interval that can be investigate in acetate buffer is, however, not great, since in low concentrations boundary anomalies occur, whereas at high concentrations the voltage on the apparatus must be taken low in order to avoid too great heat effects, and the measurements therefore become inaccurate.

Two experiments were made in acetate buffer of 0.05 n. sodium acetate concentration (tables XVII and XVIII).

Table XVII.

Exp. 10. Sodium acetate 0.05 n. Acetic acid 0.116 n.  $p_H = 4.28$ .  $\kappa = 3.36 \times 10^{-3}$ .  
Migration cathodic.

| $i \times 10^3$ | $t$ | $A_L$ | $A_R$ | $A_M$ | $u_{20} \times 10^5$ |
|-----------------|-----|-------|-------|-------|----------------------|
| 1.970           | 65  | -0.74 | +0.80 | 0.77  | 2.56                 |
| 1.902           | 62  | -0.72 | +0.64 | 0.68  | 2.46                 |
| 1.885           | 60  | -0.78 | +0.56 | 0.67  | 2.53                 |
| 1.864           | 62  | -0.78 | +0.92 | 0.85  | 3.14                 |
|                 |     |       |       |       | 2.7                  |

Table XVIII.

Exp. 11. Sodium acetate 0.05 n. Acetic acid 0.047 n.  $p_H = 4.67$ .  $\kappa = 3.36 \times 10^{-3}$ .  
Migration anodic.

| $i \times 10^3$ | $t$ | $A_L$ | $A_R$ | $A_M$ | $u_{20} \times 10^5$ |
|-----------------|-----|-------|-------|-------|----------------------|
| 2.103           | 62  | +0.20 | —     | 0.20  | 0.65                 |
| 2.092           | 69  | +0.20 | -0.20 | 0.20  | 0.60                 |
| 2.060           | 60  | +0.26 | -0.34 | 0.30  | 1.04                 |
| 1.995           | 60  | +0.18 | -0.28 | 0.23  | 0.82                 |
|                 |     |       |       |       | 0.8                  |

Evidently, the isoelectric point remains almost unchanged ( $p_H^\circ = 4.58$ ), whereas  $\frac{du}{dp_H}$  is probably decreased somewhat (to  $9 \times 10^{-5}$ ).

*Serum albumin.* The protein concentration was 0.23 %. The exposures were taken in short-wave ultraviolet.



Table XIX.

Exp. 1. Sodium acetate 0.02 n. Acetic acid 0.052 n.  $p_H = 4.21$ . Migration cathodic.

| $i \times 10^3$ | $t$ | $\Delta_L$ | $\Delta_R$ | $\Delta_M$ | $u_{20} \times 10^5$ |
|-----------------|-----|------------|------------|------------|----------------------|
| 1.500           | 60  | -3.10      | +2.90      | 3.00       | 6.13                 |
| 1.500           | 80  | -3.60      | +3.80      | 3.70       | 5.67                 |
| 1.501           | 60  | +3.10      | -2.90      | 3.00       | <u>6.13</u>          |
|                 |     |            |            |            | 6.0                  |

Table XX.

Exp. 2. Sodium acetate 0.02 n. Acetic acid 0.039 n.  $p_H = 4.40$ . Migration cathodic.

| $i \times 10^3$ | $t$ | $\Delta_L$ | $\Delta_R$ | $\Delta_M$ | $u_{20} \times 10^5$ |
|-----------------|-----|------------|------------|------------|----------------------|
| 1.492           | 30  | -1.10      | +1.06      | 1.08       | 4.41                 |
| 1.492           | 45  | +1.68      | -1.60      | 1.64       | 4.46                 |
| 1.495           | 120 | -4.24      | +4.20      | 4.22       | <u>4.30</u>          |
|                 |     |            |            |            | 4.4                  |

Table XXI.

Exp. 3. Sodium acetate 0.02 n. Acetic acid 0.02 n.  $p_H = 4.68$ . Migration cathodic.

| $i \times 10^3$ | $t$ | $\Delta_L$ | $\Delta_R$ | $\Delta_M$ | $u_{20} \times 10^5$ |
|-----------------|-----|------------|------------|------------|----------------------|
| 1.485           | 60  | +0.92      | -1.04      | 0.98       | 2.01                 |
| 1.485           | 120 | -2.00      | +1.92      | 1.96       | 2.01                 |
| 1.480           | 60  | +1.02      | -0.98      | 1.00       | <u>2.05</u>          |
|                 |     |            |            |            | 2.0                  |

Table XXII.

Exp. 4. Sodium acetate 0.02 n. Acetic acid 0.01 n.  $p_H = 4.95$ . Migration anodic.

| $i \times 10^3$ | $t$ | $\Delta_L$ | $\Delta_R$ | $\Delta_M$ | $u_{20} \times 10^5$ |
|-----------------|-----|------------|------------|------------|----------------------|
| 1.382           | 120 | +0.68      | -0.44      | 0.56       | 0.62                 |
| 1.382           | 240 | -1.62      | +0.96      | 1.29       | <u>0.71</u>          |
|                 |     |            |            |            | 0.7                  |

Table XXIII.

Exp. 5. Sodium acetate 0.02 n. Acetic acid 0.0064 n.  $p_H = 5.13$ . Migration anodic.

| $i \times 10^3$ | $t$ | $\Delta_L$ | $\Delta_R$ | $\Delta_M$ | $u_{20} \times 10^5$ |
|-----------------|-----|------------|------------|------------|----------------------|
| 1.475           | 120 | -2.34      | +2.38      | 2.36       | 2.44                 |
| 1.475           | 150 | -3.28      | +2.80      | 3.04       | 2.51                 |
| 1.479           | 62  | +1.18      | —          | 1.18       | 2.35                 |
|                 |     |            |            |            | 2.4                  |

Table XXIV.

Exp. 6. Sodium acetate 0.02 n. Acetic acid 0.004 n.  $p_H = 5.84$ . Migration anodic.

| $i \times 10^3$ | $t$ | $d_L$ | $d_R$ | $d_M$ | $u_{20} \times 10^5$ |
|-----------------|-----|-------|-------|-------|----------------------|
| 1.486           | 60  | -1.98 | +2.02 | 2.00  | 4.10                 |
| 1.486           | 60  | —     | +2.12 | 2.12  | 4.85                 |
| 1.476           | 90  | +2.82 | -3.36 | 3.10  | 4.25                 |
|                 |     |       |       |       | 4.2                  |

Fig. 19 gives the curves from exp. 3, and the diagram fig. 20 the graphical representation of the results. The isoelectric point is  $p_H^\circ = 4.88$ .  $\frac{du}{dp_H} = 9.1 \times 10^{-5}$  in the isoelectric point.

It proved necessary to use only freshly prepared serum albumin. Still the migration was never quite uniform, especially not in exp. 4.

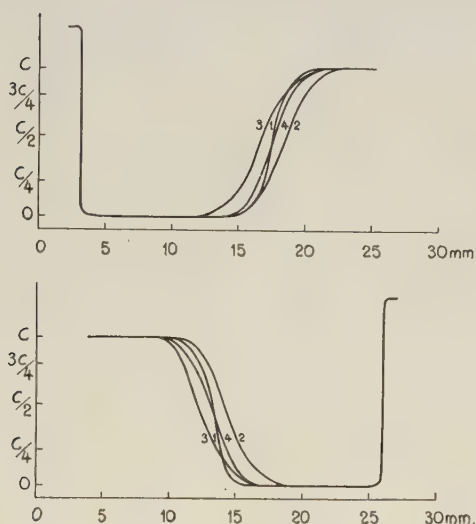


Fig. 19.

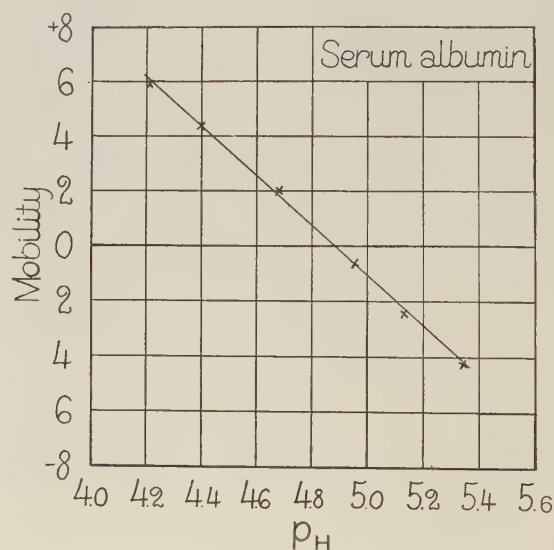


Fig. 20.

*R-Phycoerythrin.* This protein precipitates partly from its solution in the neighbourhood of the isoelectric point. Solutions of 0.2% concentration keep clear at  $p_H$  values above 4.6 but still form some precipitate at  $p_H = 3.3$ . For the determinations long-wave ultraviolet light was used. The protein concentration was 0.2%.

On the acid side of the isoelectric point the solutions had to be filtered to remove the amount of precipitate formed. In the first two experiments this amount was very small; in the third, however, the main part of the protein was precipitated. The filtrate showed a protein concentration (determined with a colorimeter) of only 0.05%.

Table XXV.

Exp. 1. Sodium acetate 0.02 n. Acetic acid 0.4 n.  $p_H = 3.34$ . Migration cathodic.

| $i \times 10^3$ | $t$ | $A_L$   | $A_R$  | $A_M$ | $u_{20} \times 10^5$ |
|-----------------|-----|---------|--------|-------|----------------------|
| 2.093           | 60  | -10.10  | + 9.98 | 10.04 | 15.63                |
| 2.093           | 120 | + 20.22 | -20.12 | 20.17 | <u>15.70</u>         |
|                 |     |         |        |       | 15.7                 |

Table XXVI.

Exp. 2. Sodium acetate 0.02 n. Acetic acid 0.2 n.  $p_H = 3.67$ . Migration cathodic.

| $i \times 10^3$ | $t$ | $A_L$  | $A_R$  | $A_M$ | $u_{20} \times 10^5$ |
|-----------------|-----|--------|--------|-------|----------------------|
| 1.263           | 60  | -3.38  | + 3.40 | 3.39  | 8.46                 |
| 1.263           | 60  | -3.52  | + 3.36 | 3.44  | 8.58                 |
| 1.263           | 120 | + 7.10 | -6.76  | 6.93  | <u>8.64</u>          |
|                 |     |        |        |       | 8.6                  |

Table XXVII.

Exp. 3. Sodium acetate 0.02 n. Acetic acid 0.1 n.  $p_H = 3.94$ . Migration cathodic.

| $i \times 10^3$ | $t$ | $A_L$  | $A_R$  | $A_M$ | $u_{20} \times 10^5$ |
|-----------------|-----|--------|--------|-------|----------------------|
| 1.237           | 120 | -3.96  | + 3.62 | 3.80  | 4.81                 |
| 1.237           | 180 | + 5.70 | -6.18  | 5.94  | <u>5.02</u>          |
|                 |     |        |        |       | 4.9                  |

Table XXVIII.

Exp. 4. Sodium acetate 0.02 n. Acetic acid 0.021 n.  $p_H = 4.63$ . Migration anodic.

| $i \times 10^3$ | $t$ | $A_L$ | $A_R$  | $A_M$ | $u_{20} \times 10^5$ |
|-----------------|-----|-------|--------|-------|----------------------|
| 0.312           | 120 | +1.16 | -0.86  | 1.01  | 4.93                 |
| 1.504           | 45  | +1.60 | -1.76  | 1.68  | 4.54                 |
| 1.530           | 100 | -3.98 | + 4.16 | 4.07  | <u>4.86</u>          |
|                 |     |       |        |       | 4.8                  |

Table XXIX.

Exp. 5. Sodium acetate 0.02 n. Acetic acid 0.012 n.  $p_H = 4.90$ . Migration anodic.

| $i \times 10^3$ | $t$ | $A_L$  | $A_R$  | $A_M$ | $u_{20} \times 10^5$ |
|-----------------|-----|--------|--------|-------|----------------------|
| 1.390           | 45  | -2.74  | + 3.30 | 3.02  | 8.82                 |
| 1.393           | 60  | + 4.10 | -3.44  | 3.77  | 8.24                 |
| 0.925           | 60  | + 2.46 | -2.78  | 2.62  | <u>8.63</u>          |
|                 |     |        |        |       | 8.6                  |



Table XXX.

Exp. 6. Sodium acetate 0.02 n. Acetic acid 0.003 n.  $p_H = 5.42$  (buffer 5.43, protein 5.41). Migration anodic.

| $i \times 10^3$ | $t$ | $\Delta_L$ | $\Delta_R$ | $\Delta_M$ | $u_{20} \times 10^5$ |
|-----------------|-----|------------|------------|------------|----------------------|
| 1.661           | 30  | -3.76      | +3.90      | 3.83       | 14.05                |
| 1.661           | 30  | +3.80      | -4.20      | 4.00       | 14.67                |
| 1.661           | 25  | -3.12      | +3.02      | 3.07       | 13.51                |
|                 |     |            |            |            | 14.1                 |

Fig. 21 shows the curves from exp. 2, and fig. 22 the graphical representation of the results. The isoelectric point is situated at  $p_H^\circ = 4.25$ . Here  $\frac{du}{dp_H} = 14.2 \times 10^{-5}$ .

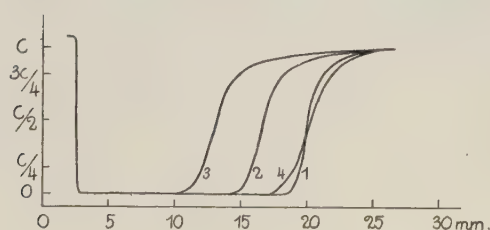


Fig. 21.

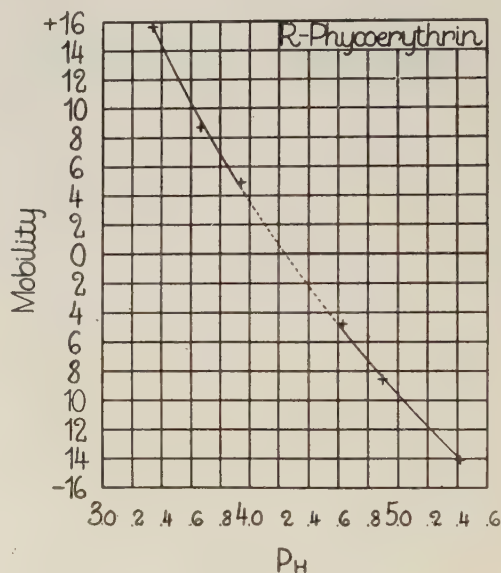


Fig. 22.

The migration was uniform, even in the most acid solutions, with almost congruent boundaries.

*Helix Hemocyanin.* The protein concentration was 0.15 %. Short-wave ultra-violet light was used.

Table XXXI.

Exp. 1. Sodium acetate 0.02 n. Acetic acid 0.04 n.  $p_H = 4.36$ . Migration cathodic.

| $i \times 10^3$ | $t$ | $\Delta_L$ | $\Delta_R$ | $\Delta_M$ | $u_{20} \times 10^5$ |
|-----------------|-----|------------|------------|------------|----------------------|
| 1.200           | 60  | -2.84      | +2.24      | 2.29       | 5.81                 |
| 1.200           | 90  | +3.36      | -3.48      | 3.42       | 5.78                 |
| 1.202           | 60  | +2.26      | -2.36      | 2.31       | 5.85                 |
| 1.204           | 124 | -4.82      | +4.74      | 4.78       | 5.85                 |
|                 |     |            |            |            | 5.8                  |

Table XXXII.

Exp. 2. Sodium acetate 0.02 n. Acetic acid 0.02 n.  $p_H = 4.67$ . Migration cathodic.

| $i \times 10^3$ | $t$ | $\Delta_L$ | $\Delta_R$ | $\Delta_M$ | $u_{20} \times 10^5$ |
|-----------------|-----|------------|------------|------------|----------------------|
| 1.280           | 60  | -1.14      | +1.24      | 1.19       | 2.83                 |
| 1.280           | 60  | +1.32      | -1.20      | 1.26       | 2.99                 |
| 1.280           | 60  | +1.20      | -1.26      | 1.23       | <u>2.92</u>          |
|                 |     |            |            |            | 2.9                  |

Table XXXIII.

Exp. 3. Sodium acetate 0.02 n. Acetic acid 0.008 n.  $p_H = 5.07$ . Migration anodic.

| $i \times 10^3$ | $t$ | $\Delta_L$              | $\Delta_R$ | $\Delta_M$ | $u_{20} \times 10^5$ |
|-----------------|-----|-------------------------|------------|------------|----------------------|
| 1.221           | 60  | to small to be measured |            |            | —                    |
| 1.199           | 381 | +0.38                   | -0.38      | 0.38       | 0.15                 |
|                 |     |                         |            |            | <u>0.2</u>           |

Table XXXIV.

Exp. 4. Sodium acetate 0.02 n. Acetic acid 0.0036 n.  $p_H = 5.39$ . Migration anodic.

| $i \times 10^3$ | $t$ | $\Delta_L$ | $\Delta_R$ | $\Delta_M$ | $u_{20} \times 10^5$ |
|-----------------|-----|------------|------------|------------|----------------------|
| 1.234           | 60  | +0.94      | -0.94      | 0.94       | 2.32                 |
| 1.234           | 90  | +1.40      | -1.40      | 1.40       | 2.30                 |
| 1.238           | 535 | -8.18      | +8.30      | 8.24       | <u>2.27</u>          |
|                 |     |            |            |            | 2.3                  |

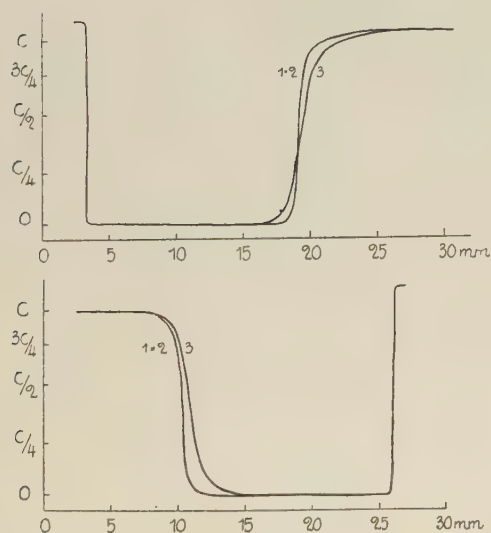


Fig. 23.

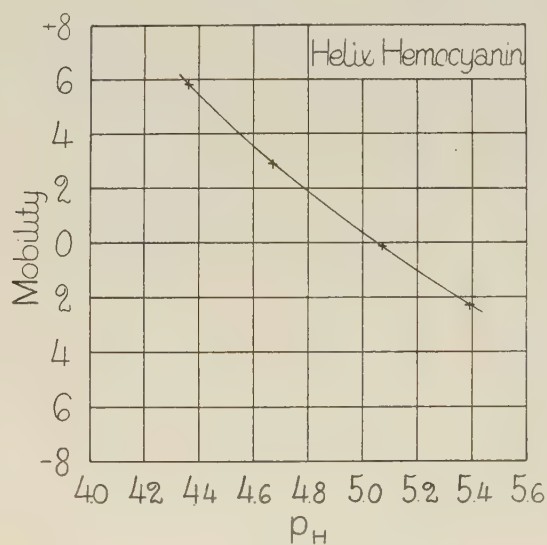


Fig. 24.

Fig. 23 gives the curves from experiment 3. A graphical representation of the results is given in fig. 24. The isoelectric point is  $p_H^{\circ} = 5.05$ .  $\frac{du}{dp_H} = 7.3 \times 10^{-5}$ .

We find from the tables that the values obtained in an experiment for different time intervals and in the two different tubes agree very well, better than for any other protein studied. This is probably due in the first place to the very low diffusion constant of hemocyanin ( $D = 1.78 \times 10^{-7}$ , whereas for egg albumin  $D = 9.6 \times 10^{-7}$  (72)). The boundaries remain very sharp during the experiment. From this it is also evident that the hemocyanin must be of a very high degree of uniformity.

The sharpness of the boundaries made it possible to measure the mobility in a  $p_H$  distance of only 0.02 from the isoelectric point (exp. 3).

*C-Phycocyan.* Protein concentration 0.18—0.20 %. Long-wave ultraviolet was used.

Table XXXV.

Exp. 1. Sodium acetate 0.02 n. Acetic acid 0.08 n.  $p_H = 4.07$ . Migration cathodic.

| $i \times 10^8$ | $t$ | $A_L$ | $A_R$ | $A_M$ | $u_{20} \times 10^5$ |
|-----------------|-----|-------|-------|-------|----------------------|
| 1.339           | 60  | -3.40 | +3.26 | 3.33  | 7.73                 |
| 1.340           | 60  | -3.36 | +3.04 | 3.20  | 7.43                 |
| 1.331           | 60  | +3.36 | -2.94 | 3.15  | 7.36                 |
| 1.323           | 60  | +3.28 | -3.44 | 3.36  | <u>7.90</u>          |
|                 |     |       |       |       | 7.6                  |

Table XXXVI.

Exp. 2. Sodium acetate 0.02 n. Acetic acid 0.044 n.  $p_H = 4.34$ . Migration cathodic.

| $i \times 10^8$ | $t$ | $A_L$ | $A_R$ | $A_M$ | $u_{20} \times 10^5$ |
|-----------------|-----|-------|-------|-------|----------------------|
| 1.331           | 60  | -1.74 | +2.00 | 1.87  | 4.28                 |
| 1.321           | 60  | -1.92 | +1.82 | 1.87  | 4.31                 |
| 1.322           | 180 | +5.84 | -5.98 | 5.91  | 4.54                 |
|                 |     |       |       |       | 4.4                  |

Table XXXVII.

Exp. 3. Sodium acetate 0.02 n. Acetic acid 0.02 n.  $p_H = 4.68$ . Migration cathodic.

| $i \times 10^8$ | $t$ | $A_L$ | $A_R$ | $A_M$ | $u_{20} \times 10^5$ |
|-----------------|-----|-------|-------|-------|----------------------|
| 1.420           | 120 | -0.78 | —     | 0.78  | 0.84                 |
| 1.420           | 180 | +0.86 | -0.84 | 0.85  | <u>0.61</u>          |
|                 |     |       |       |       | 0.7                  |



Table XXXVIII.

Exp. 4. Sodium acetate 0.02 n. Acetic acid 0.012 n.  $p_H = 4.87$ . Migration anodic.

| $i \times 10^8$ | $t$ | $A_L$ | $A_R$ | $A_M$ | $u_{20} \times 10^5$ |
|-----------------|-----|-------|-------|-------|----------------------|
| 1.480           | 180 | +1.20 | -1.24 | 1.22  | 0.87                 |
| 1.480           | 192 | -1.62 | +1.44 | 1.53  | <u>1.02</u>          |
|                 |     |       |       |       | 0.9                  |

Table XXXIX.

Exp. 5. Sodium acetate 0.02 n. Acetic acid 0.01 n.  $p_H = 4.97$ . Migration anodic.

| $i \times 10^8$ | $t$ | $A_L$ | $A_R$ | $A_M$ | $u_{20} \times 10^5$ |
|-----------------|-----|-------|-------|-------|----------------------|
| 1.363           | 60  | +0.70 | -0.80 | 0.75  | 1.68                 |
| 1.362           | 60  | +0.66 | -0.78 | 0.71  | 1.61                 |
| 1.363           | 60  | -0.66 | +0.76 | 0.71  | 1.59                 |
| 1.363           | 60  | -0.70 | +0.68 | 0.69  | <u>1.54</u>          |
|                 |     |       |       |       | 1.6                  |

Table XL.

Exp. 6. Sodium acetate 0.02 n. Acetic acid 0.004 n.  $p_H = 5.35$ . Migration anodic.

| $i \times 10^8$ | $t$ | $A_L$ | $A_R$ | $A_M$ | $u_{20} \times 10^5$ |
|-----------------|-----|-------|-------|-------|----------------------|
| 1.488           | 60  | +2.12 | -1.96 | 2.04  | 4.18                 |
| 1.500           | 60  | +1.68 | -1.72 | 1.70  | 3.45                 |
| 1.488           | 60  | -1.68 | +1.72 | 1.70  | <u>3.48</u>          |
|                 |     |       |       |       | 3.7                  |

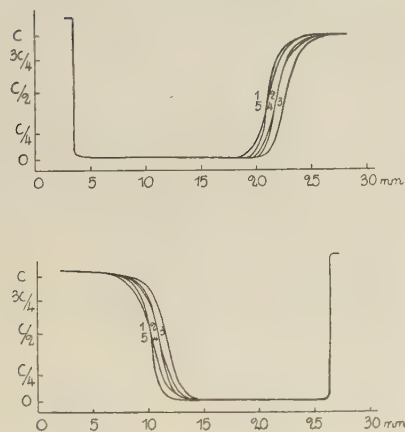


Fig. 25.

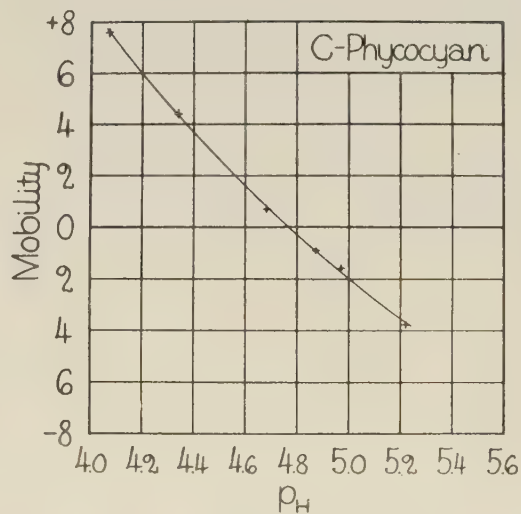


Fig. 26.

The curves from exp. 5 are given in fig. 25. The results are collected in the diagram fig. 26. The isoelectric point is  $p_H^0 = 4.76$ . Here  $\frac{du}{dp_H} = 9.1 \times 10^{-5}$ .

It should be noted that, unlike phycoerythrin, this protein is not precipitated at the isoelectric point.

The experiments were made with material that had been recrystallized (see above p. 50). Non-crystallized material gives different results: the isoelectric point was 4.67 and the slope of the mobility- $p_H$  curve was smaller ( $\frac{du}{dp_H} = 8.0 \times 10^{-5}$ ). The migration of this material was non-uniform (see below p. 94).

The migration of the crystallized material was uniform.

*Bence Jones protein.* Protein concentration 0.15 %. Short-wave ultraviolet was used.

Table XLI.

| Exp. 1. Sodium acetate 0.02 n. Acetic acid 0.04 n. $p_H = 4.37$ . Migration cathodic. |     |       |       |       |                      |
|---------------------------------------------------------------------------------------|-----|-------|-------|-------|----------------------|
| $i \times 10^3$                                                                       | $t$ | $A_L$ | $A_R$ | $A_M$ | $u_{20} \times 10^5$ |
| 1.210                                                                                 | 180 | -5.50 | +5.18 | 5.34  | 4.48                 |
| 1.203                                                                                 | 120 | +3.64 | -4.24 | 3.94  | 4.99                 |
| 1.208                                                                                 | 120 | +3.96 | -3.56 | 3.76  | <u>4.74</u>          |
|                                                                                       |     |       |       |       | 4.7                  |

Table XLII.

| Exp. 2. Sodium acetate 0.02 n. Acetic acid 0.02 n. $p_H = 4.67$ . Migration cathodic. |     |       |       |       |                      |
|---------------------------------------------------------------------------------------|-----|-------|-------|-------|----------------------|
| $i \times 10^3$                                                                       | $t$ | $A_L$ | $A_R$ | $A_M$ | $u_{20} \times 10^5$ |
| 1.185                                                                                 | 60  | -1.08 | +1.06 | 1.07  | 2.75                 |
| 1.185                                                                                 | 120 | -2.18 | +2.60 | 2.39  | 3.07                 |
| 1.176                                                                                 | 120 | +2.18 | -2.60 | 2.39  | 3.10                 |
| 1.178                                                                                 | 120 | +2.38 | -2.04 | 2.21  | <u>2.86</u>          |
|                                                                                       |     |       |       |       | 2.9                  |

Table XLIII.

| Exp. 3. Sodium acetate 0.02 n. Acetic acid 0.004 n. $p_H = 5.34$ . Migration anodic. |     |       |       |       |                      |
|--------------------------------------------------------------------------------------|-----|-------|-------|-------|----------------------|
| $i \times 10^3$                                                                      | $t$ | $A_L$ | $A_R$ | $A_M$ | $u_{20} \times 10^5$ |
| 1.160                                                                                | 180 | +0.88 | -1.20 | 1.04  | 0.91                 |
| 1.151                                                                                | 120 | -0.74 | +0.64 | 0.69  | 0.91                 |
| 1.153                                                                                | 120 | -0.68 | +0.72 | 0.70  | <u>0.92</u>          |
|                                                                                      |     |       |       |       | 0.9                  |

Table XLIV.

Exp. 4. Sodium acetate 0.02 n. Acetic acid 0.002 n.  $p_H = 5.63$  for the buffer, 5.63 for the prot. Mean  $p_H = 5.65$ . Migration anodic.

| $i \times 10^3$ | $t$ | $\Delta_L$ | $\Delta_R$ | $\Delta_M$ | $u_{20} \times 10^5$ |
|-----------------|-----|------------|------------|------------|----------------------|
| 1.420           | 120 | +2.20      | -2.56      | 2.38       | 2.55                 |
| 1.420           | 120 | -2.50      | +2.50      | 2.50       | 2.68                 |
| 1.420           | 60  | +1.10      | -1.20      | 1.15       | 2.47                 |
|                 |     |            |            |            | 2.6                  |

Fig. 27 shows the curves from exp. 4. The results are collected in the diagram fig. 28.

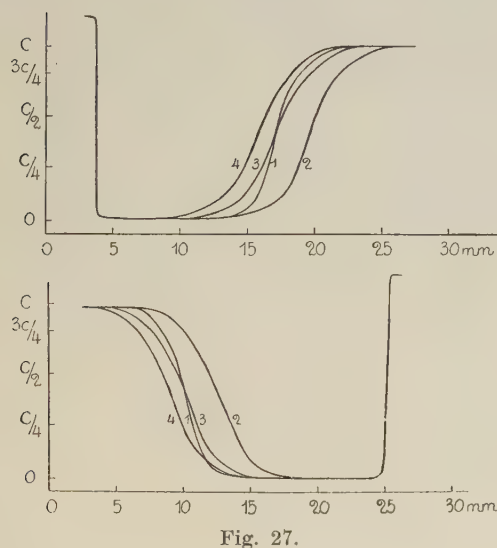


Fig. 27.

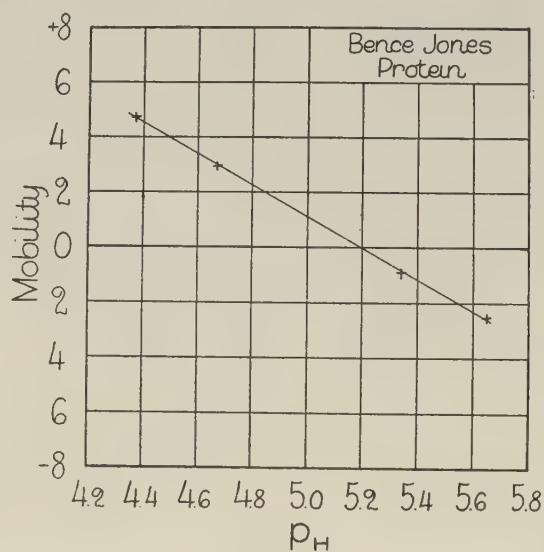


Fig. 28.

The isoelectric point is  $p_H^0 = 5.20$ .  $\frac{du}{dp_H} = 5.8 \times 10^{-5}$ . The migration was not very regular, but no definite evidence of non-uniformity could be obtained.

### Experiments in barium acetate-acetic acid buffers.

Egg albumin and phycoerythrin were investigated. The protein material and concentrations were the same as were used in the experiments in sodium acetate buffers.



*Egg albumin.*

Table XLV.

Exp. 1. Barium acetate 0.02 n. Acetic acid 0.05 n.  $p_H = 4.27$ .  $\kappa = 1.52 \times 10^{-3}$ .  
Migration cathodic.

| $i \times 10^3$ | $t$ | $A_L$ | $A_R$ | $A_M$ | $u_{20} \times 10^5$ |
|-----------------|-----|-------|-------|-------|----------------------|
| 1.550           | 120 | +3.32 | -3.18 | 3.25  | 3.37                 |
| 1.568           | 215 | -7.18 | +6.52 | 6.85  | 3.92                 |
|                 |     |       |       |       | 3.6                  |

Table XLVI.

Exp. 2. Barium acetate 0.02 n. Acetic acid 0.0262 n.  $p_H = 4.51$ .  $\kappa = 1.51 \times 10^{-3}$ .  
Migration cathodic.

| $i \times 10^3$ | $t$ | $A_L$ | $A_R$ | $A_M$ | $u_{20} \times 10^5$ |
|-----------------|-----|-------|-------|-------|----------------------|
| 1.545           | 90  | -0.58 | +0.70 | 0.64  | 0.88                 |
| 1.540           | 183 | +1.80 | -1.50 | 1.65  | 1.12                 |
| 1.546           | 120 | -1.34 | +1.16 | 1.25  | 1.29                 |
|                 |     |       |       |       | 1.1                  |

Table XLVII.

Exp. 3. Barium acetate 0.02 n. Acetic acid 0.008 n.  $p_H = 5.11$ .  $\kappa = 1.50 \times 10^{-3}$ .  
Migration anodic.

| $i \times 10^3$ | $t$ | $A_L$ | $A_R$ | $A_M$ | $u_{20} \times 10^5$ |
|-----------------|-----|-------|-------|-------|----------------------|
| 1.510           | 60  | +1.46 | -1.66 | 1.56  | 3.27                 |
| 1.506           | 60  | +1.44 | -1.28 | 1.36  | 2.87                 |
| 1.506           | 107 | -2.42 | +2.44 | 2.43  | 2.87                 |
|                 |     |       |       |       | 3.0                  |

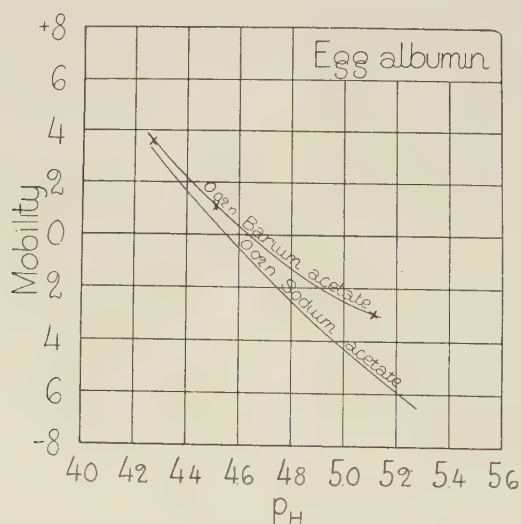


Fig. 29.

The result is shown in the diagram fig. 29. For purposes of comparison the curve obtained for 0.02 n sodium acetate buffer is also given. The isoelectric point in barium acetate buffer is evidently  $p_H^0 = 4.63$ , and the shape of the curve has been changed, corresponding to depressed mobilities on the negative side.

*Phycoerythrin.*

*Table XLVIII.*

Exp. 1. Barium acetate 0.02 n. Acetic acid 0.4 n.  $p_H = 3.40$ .  $\kappa = 1.57 \times 10^{-3}$ .  
Migration cathodic.

| $i \times 10^3$ | $t$ | $A_L$ | $A_R$ | $A_M$ | $u_{20} \times 10^5$ |
|-----------------|-----|-------|-------|-------|----------------------|
| 1.276           | 60  | -4.30 | +4.38 | 4.34  | 11.30                |
| 1.268           | 120 | +8.88 | -8.62 | 8.75  | 11.46                |
| 1.277           | 60  | -4.58 | +4.24 | 4.41  | 11.47                |
|                 |     |       |       |       | 11.4                 |

*Table XLIX.*

Exp. 2. Barium acetate 0.02 n. Acetic acid 0.1 n.  $p_H = 4.01$ .  $\kappa = 1.54 \times 10^{-3}$ .  
Migration cathodic.

| $i \times 10^3$ | $t$ | $A_L$ | $A_R$ | $A_M$ | $u_{20} \times 10^5$ |
|-----------------|-----|-------|-------|-------|----------------------|
| 1.230           | 125 | -2.56 | —     | 2.56  | 3.25                 |
| 1.254           | 240 | +5.90 | -6.06 | 5.98  | <u>3.88</u>          |
|                 |     |       |       |       | 3.6                  |

*Table L.*

Exp. 3. Barium acetate 0.02 n. Acetic acid 0.0202 n.  $p_H = 4.71$ .  $\kappa = 1.51 \times 10^{-3}$ .  
Migration anodic.

| $i \times 10^3$ | $t$ | $A_L$ | $A_R$ | $A_M$ | $u_{20} \times 10^5$ |
|-----------------|-----|-------|-------|-------|----------------------|
| 1.232           | 160 | +4.06 | -3.94 | 4.00  | 3.89                 |
| 1.234           | 75  | -1.96 | +2.00 | 1.98  | 4.10                 |
| 1.240           | 82  | -2.10 | +1.96 | 2.03  | <u>3.83</u>          |
|                 |     |       |       |       | 3.9                  |

*Table LI.*

Exp. 4. Barium acetate 0.02 n. Acetic acid 0.0062 n.  $p_H = 5.22$ .  $\kappa = 1.49 \times 10^{-3}$ .  
Migration anodic.

| $i \times 10^3$ | $t$ | $A_L$ | $A_R$ | $A_M$ | $u_{20} \times 10^5$ |
|-----------------|-----|-------|-------|-------|----------------------|
| 1.213           | 120 | +5.36 | -5.36 | 5.36  | 6.96                 |
| 1.213           | 120 | -5.50 | +5.18 | 5.34  | 6.94                 |
| 1.213           | 122 | -5.24 | +5.52 | 5.38  | <u>6.88</u>          |
|                 |     |       |       |       | 6.9                  |

In exp. 2 and 3 the protein concentration was lower than 0.2 %. The resulting mobility curve is given in fig. 30. Evidently the  $\text{Ba}^{++}$  ions act depressing upon the negative mobilities and also cause a small change in the isoelectric point to  $p_H^\circ = 4.30$ .

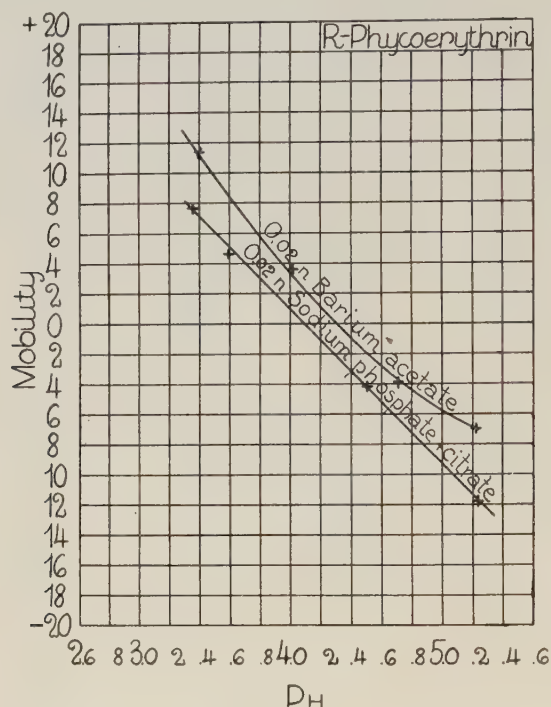


Fig. 30.

### Experiments in sodium phosphate-citric acid buffers.

Only phycoerythrin was investigated. Protein concentration 0.2 %.

Table LII.

Exp. 1.  $\text{Na}_2\text{HPO}_4$  0.01 m. Citric acid 0.015 m.  $p_H = 3.35$ .  $\alpha = 1.37 \times 10^{-3}$ .  
Migration cathodic.

| $i \times 10^3$ | $t$ | $\Delta_L$ | $\Delta_R$ | $\Delta_M$ | $u_{20} \times 10^5$ |
|-----------------|-----|------------|------------|------------|----------------------|
| 1.880           | 120 | -8.60      | +8.64      | 8.62       | 6.64                 |
| 1.877           | 121 | +8.56      | -8.80      | 8.68       | 6.65                 |
| 1.879           | 60  | -4.34      | +4.40      | 4.37       | 6.74                 |
|                 |     |            |            |            | 6.7                  |



Table LIII.

Exp. 2.  $\text{Na}_2\text{HPO}_4$  0.01 m. Citric acid 0.012 m.  $p_H = 3.59$ .  $\kappa = 1.36 \times 10^{-3}$ .  
Migration cathodic.

| $i \times 10^8$ | $t$ | $\Delta_L$ | $\Delta_R$ | $\Delta_M$ | $u_{20} \times 10^5$ |
|-----------------|-----|------------|------------|------------|----------------------|
| 1.815           | 60  | -3.04      | +3.00      | 3.02       | 4.79                 |
| 1.820           | 120 | +6.02      | -6.10      | 6.06       | 4.79                 |
| 1.820           | 60  | -2.80      | +3.04      | 2.92       | <u>4.62</u>          |
|                 |     |            |            |            | 4.7                  |

Table LIV.

Exp. 3.  $\text{Na}_2\text{HPO}_4$  0.01 m. Citric acid 0.0069 m.  $p_H = 4.50$ .  $\kappa = 1.35 \times 10^{-3}$ .  
Migration anodic.

| $i \times 10^8$ | $t$ | $\Delta_L$ | $\Delta_R$ | $\Delta_M$ | $u_{20} \times 10^5$ |
|-----------------|-----|------------|------------|------------|----------------------|
| 1.650           | 120 | +4.76      | -4.88      | 4.82       | 4.17                 |
| 1.660           | 120 | -4.94      | +4.88      | 4.91       | 4.22                 |
| 1.660           | 120 | -4.20      | +5.14      | 4.67       | <u>4.02</u>          |
|                 |     |            |            |            | 4.1                  |

Table LV.

Exp. 4.  $\text{Na}_2\text{HPO}_4$  0.01 m. Citric acid 0.005 m.  $p_H = 5.23$ .  $\kappa = 1.35 \times 10^{-3}$ .

| $i \times 10^8$ | $t$ | $\Delta_L$ | $\Delta_R$ | $\Delta_M$ | $u_{20} \times 10^5$ |
|-----------------|-----|------------|------------|------------|----------------------|
| 1.621           | 60  | +6.86      | -6.66      | 6.76       | 11.91                |
| 1.616           | 120 | -13.62     | +13.20     | 13.41      | 11.85                |
| 1.616           | 60  | +6.76      | -6.28      | 6.52       | 11.52                |
|                 |     |            |            |            | 11.8                 |

Exp. 2. and especially exp. 3 were made with a solution of protein concentration lower than 0.2 %. A graphical representation of the results is given in fig. 30, which also contains the mobility curve in the barium acetate buffer solutions investigated. It is seen that in this case the positive mobilities are decreased and the position of the isoelectric point is shifted to  $p_H^\circ = 4.09$ .

### Experiments in phosphate buffer.

Although the present investigation was planned to concern mainly the electrophoresis in the isoelectric region it was considered to be of some interest, especially as regards uniformity (see further p. 96), to make a few determinations at higher mobilities in the  $p_H$  region of neutral reaction.

*Egg albumin.* Three experiments with different  $p_H$ -values and constant ionic strength = 0.02 were made (tables LVI—LVIII).

Table LVI.

Exp. 1.  $\text{KH}_2\text{PO}_4$  0.015 m.  $\text{Na}_2\text{HPO}_4$  0.00167 m.  $p_H = 5.98$ .  $\kappa = 1.57 \times 10^{-3}$ .  
Migration anodic.

| $i \times 10^3$ | $t$ | $A_L$ | $A_R$ | $A_M$ | $u_{20} \times 10^5$ |
|-----------------|-----|-------|-------|-------|----------------------|
| 1.301           | 30  | +2.22 | -2.08 | 2.15  | 10.98                |
| 1.299           | 30  | +2.14 | -2.10 | 2.12  | 10.84                |
| 2.578           | 30  | -4.36 | +4.48 | 4.42  | 11.39                |
| 2.578           | 30  | -4.04 | +4.18 | 4.11  | 10.59                |
|                 |     |       |       |       | 11.0                 |

Table LVII.

Exp. 2.  $\text{KH}_2\text{PO}_4$  0.005 m.  $\text{Na}_2\text{HPO}_4$  0.005 m.  $p_H = 6.93$ .  $\kappa = 1.24 \times 10^{-3}$ .  
Migration anodic.

| $i \times 10^3$ | $t$ | $A_L$ | $A_R$ | $A_M$ | $u_{20} \times 10^5$ |
|-----------------|-----|-------|-------|-------|----------------------|
| 1.039           | 30  | +3.06 | -3.00 | 3.03  | 15.30                |
| 1.039           | 30  | —     | -2.78 | 2.78  | 14.04                |
| 2.041           | 30  | —     | +5.46 | 5.46  | 14.03                |
| 2.054           | 30  | -5.52 | +6.36 | 5.94  | 15.17                |
|                 |     |       |       |       | 14.6                 |

Table LVIII.

Exp. 3.  $\text{KH}_2\text{PO}_4$  0.000714 m.  $\text{Na}_2\text{HPO}_4$  0.00643 m.  $p_H = 7.71$ .  $\kappa = 1.14 \times 10^{-3}$ .  
Migration anodic.

| $i \times 10^3$ | $t$ | $A_L$ | $A_R$ | $A_M$ | $u_{20} \times 10^5$ |
|-----------------|-----|-------|-------|-------|----------------------|
| 1.904           | 15  | +3.64 | -3.46 | 3.55  | 17.99                |
| 1.903           | 15  | —     | -3.56 | 3.56  | 18.05                |
| 1.904           | 20  | —     | +4.82 | 4.82  | 18.32                |
| 1.904           | 20  | -4.70 | +4.66 | 4.68  | 17.79                |
|                 |     |       |       |       | 18.0                 |

In exp. 2 and 3 the third exposure was spoiled by a spot on the tube.

The curves from exp. 1 are given in fig. 31. It is evident that the migration is uniform.

Evidently, in this  $p_H$ -region, the mobility still increases with  $p_H$ ; for constant ionic strength, but  $\frac{du}{dp_H}$  is smaller than in the isoelectric region.

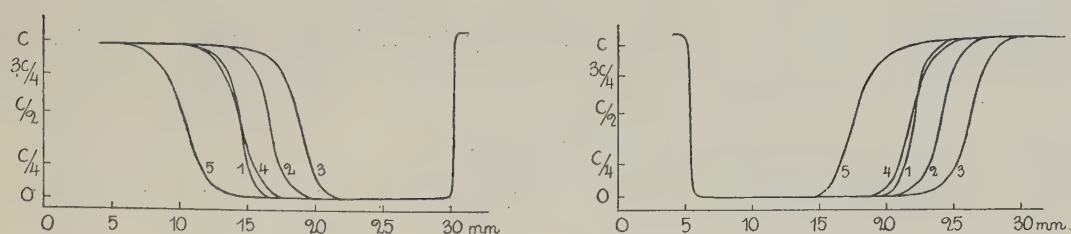


Fig. 31.

*R-Phycoerythrin.*

Table LIX.

Exp. 1.  $\text{KH}_2\text{PO}_4$  0.015 m.  $\text{Na}_2\text{HPO}_4$  0.00167 m.  $p_H = 5.98$ .  $\alpha = 1.57 \times 10^{-3}$ . Migration anodic.

| $i \times 10^3$ | $t$ | $A_L$   | $A_R$   | $A_M$ | $u_{20} \times 10^5$ |
|-----------------|-----|---------|---------|-------|----------------------|
| 1.310           | 60  | + 7.90  | — 8.06  | 7.98  | 20.22                |
| 1.310           | 120 | — 15.89 | + 16.29 | 16.09 | 20.40                |
| 1.310           | 60  | + 8.16  | — 7.88  | 8.02  | <u>20.32</u>         |
|                 |     |         |         |       | 20.3                 |

Table LX.

Exp. 2.  $\text{KH}_2\text{PO}_4$  0.005 m.  $\text{Na}_2\text{HPO}_4$  0.005 m.  $p_H = 6.93$ .  $\alpha = 1.24 \times 10^{-3}$ . Migration anodic.

| $i \times 10^3$ | $t$  | $A_L$   | $A_R$   | $A_M$ | $u_{20} \times 10^5$ |
|-----------------|------|---------|---------|-------|----------------------|
| 1.480           | 60   | — 12.14 | + 11.92 | 12.03 | 21.32                |
| 1.485           | 60.5 | + 12.14 | — 12.28 | 12.21 | 21.39                |
| 1.480           | 60   | + 12.10 | — 11.90 | 12.00 | <u>21.27</u>         |
|                 |      |         |         |       | 21.3                 |

Table LXI.

Exp. 3.  $\text{KH}_2\text{PO}_4$  0.000714 m.  $\text{Na}_2\text{HPO}_4$  0.00643 m.  $p_H = 7.72$ .  $\alpha = 1.14 \times 10^{-3}$ . Migration anodic.

| $i \times 10^3$ | $t$ | $A_L$  | $A_R$  | $A_M$ | $u_{20} \times 10^5$ |
|-----------------|-----|--------|--------|-------|----------------------|
| 1.903           | 30  | + 8.74 | — 8.86 | 8.80  | 22.28                |
| 1.903           | 30  | — 8.70 | + 8.78 | 8.74  | 22.12                |
| 1.903           | 30  | — 8.88 | + 8.72 | 8.80  | <u>22.30</u>         |
|                 |     |        |        |       | 22.2                 |

The change of mobility with  $p_H$  for phycoerythrin is evidently very small in this region. Probably a limiting value is approached.

Some experiments were also made to study the salt effect on the mobility.

Table LXII.

Exp. 4. m/60 total phosphate 1:1.  $p_H = 6.90$ .  $\kappa = 1.89 \times 10^{-3}$ . Migration anodic.

| $i \times 10^3$ | $t$ | $\Delta_L$ | $\Delta_R$ | $\Delta_M$ | $u_{20} \times 10^5$ |
|-----------------|-----|------------|------------|------------|----------------------|
| 2.259           | 15  | + 2.48     | - 2.54     | 2.51       | 17.77                |
| 0.420           | 105 | + 3.16     | - 3.34     | 3.25       | 17.68                |
| 0.420           | 137 | - 4.16     | + 4.20     | 4.18       | 17.43                |
| 2.266           | 40  | - 6.76     | + 6.82     | 6.54       | 17.31                |
|                 |     |            |            |            | 17.6                 |

Table LXIII.

Exp. 5. m/300 total phosphate 1:1.  $p_H = 7.19$ .  $\kappa = 4.63 \times 10^{-4}$ . Migration anodic.

| $i \times 10^3$ | $t$ | $\Delta_L$ | $\Delta_R$ | $\Delta_M$ | $u_{20} \times 10^5$ |
|-----------------|-----|------------|------------|------------|----------------------|
| 0.498           | 20  | + 4.24     | - 4.04     | 4.14       | 24.43                |
| 0.497           | 50  | - 10.56    | + 11.16    | 10.86      | 25.68                |
| 0.735           | 30  | + 9.46     | - 10.18    | 9.82       | 26.17                |
|                 |     |            |            |            | 25.4                 |

These two experiments together with exp. 2 demonstrate that there is a considerable influence of the salt concentration on the mobility. The migration was uniform. A diagram demonstrating the uniformity of the migration of phycoerythrin at high mobilities will be given below (p. 95).

*The other proteins.* All experiments were made in 0.01 m phosphate buffer 1:1.  $p_H = 6.93$ .  $\kappa = 1.24 \times 10^{-3}$ .

Table LXIV.

Serum albumin in 0.01 m total phosphate 1:1.  $p_H = 6.93$ . Migration anodic.

| $i \times 10^3$ | $t$ | $\Delta_L$ | $\Delta_R$ | $\Delta_M$ | $u_{20} \times 10^5$ |
|-----------------|-----|------------|------------|------------|----------------------|
| 1.039           | 30  | + 2.18     | - 2.22     | 2.20       | 11.11                |
| 1.038           | 15  | + 0.98     | - 1.34     | 1.16       | 11.72                |
| 1.039           | 60  | - 4.28     | + 4.22     | 4.25       | 10.73                |
| 2.055           | 30  | - 4.54     | + 4.32     | 4.43       | 11.31                |
|                 |     |            |            |            | 11.2                 |



Table LXV.

Helix hemocyanin in 0.01 m total phosphate 1:1.  $p_H = 6.93$ . Migration anodic.

| $i \times 10^3$ | $t$ | $\Delta_L$ | $\Delta_R$ | $\Delta_M$ | $u_{20} \times 10^5$ |
|-----------------|-----|------------|------------|------------|----------------------|
| 1.051           | 30  | +2.20      | -2.28      | 2.24       | 11.18                |
| 1.050           | 30  | +2.80      | -2.26      | 2.28       | 11.89                |
| 2.079           | 30  | -4.50      | +4.54      | 4.52       | 11.41                |
| 2.080           | 30  | -4.50      | +4.50      | 4.50       | <u>11.85</u>         |
|                 |     |            |            |            | 11.8                 |

Table LXVI.

C-Phycocyan in 0.01 m total phosphate 1:1.  $p_H = 6.94$ . Migration anodic.

| $i \times 10^3$ | $t$ | $\Delta_L$ | $\Delta_R$ | $\Delta_M$ | $u_{20} \times 10^5$ |
|-----------------|-----|------------|------------|------------|----------------------|
| 1.045           | 65  | +4.84      | -5.00      | 4.92       | 11.40                |
| 2.075           | 30  | -4.80      | +4.58      | 4.69       | 11.86                |
| 2.082           | 20  | -3.02      | +2.86      | 2.94       | <u>11.11</u>         |
|                 |     |            |            |            | 11.5                 |

Table LXVII.

Bence Jones protein in 0.01 m total phosphate 1:1.  $p_H = 6.94$ . Migration anodic.

| $i \times 10^3$ | $t$ | $\Delta_L$ | $\Delta_R$ | $\Delta_M$ | $u_{20} \times 10^5$ |
|-----------------|-----|------------|------------|------------|----------------------|
| 1.045           | 30  | +1.26      | -1.36      | 1.31       | 6.58                 |
| 1.045           | 30  | +1.22      | -1.00      | 1.11       | 5.57                 |
| 2.060           | 30  | -2.44      | +2.26      | 2.35       | 5.98                 |
| 2.090           | 30  | -2.32      | +2.30      | 2.31       | <u>5.80</u>          |
|                 |     |            |            |            | 6.0                  |

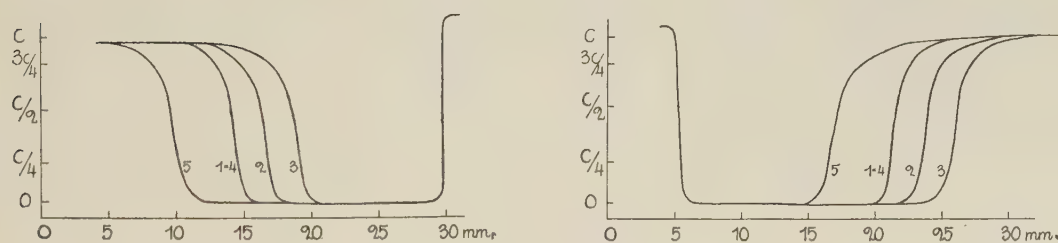


Fig. 32.

The last exposure with serum albumin, especially in the right tube, was irregular. Otherwise the migration was uniform.

The other proteins also showed uniform migration. Fig. 32 shows the curves from the experiment with hemocyanin.

The experiment with Bence Jones protein is not very accurate, since the constant concentration part of the curve was not equal for all the curves.

## 2. Experiments in unbuffered solutions.

*C-Phycocyan.* The solutions were prepared by dialyzing a 0.3% solution of phycocyan, containing 0.02 n KCl and a trace of HCl or KOH (see further the tables below) against an outer liquid containing 0.1% phycocyan and the same amount of salt. To prevent evaporation the dialysis was performed in closed bottles. The bottles with the bags were shaken several times every day.

Table LXVIII.

Exp. 1. KCl = 0.02 n. HCl originally  $2 \times 10^{-4}$  n.  $p_H$  at the end of dialysis (3 days) in inner liquid 4.27, in outer liquid 4.25, mean 4.26. Conductivity  $\kappa = 2.50 \times 10^{-3}$  and  $\kappa = 2.51 \times 10^{-3}$ , mean  $\kappa = 2.51 \times 10^{-3}$ . Migration cathodic.

| $i \times 10^3$ | $t$ | $\Delta_L$ | $\Delta_R$ | $\Delta_M$ | $u_{20} \times 10^5$ |
|-----------------|-----|------------|------------|------------|----------------------|
| 2.220           | 120 | -4.86      | +4.44      | 4.65       | 5.56                 |
| 2.220           | 60  | +2.40      | -2.24      | 2.32       | 5.54                 |
| 2.227           | 60  | +2.38      | -2.38      | 2.38       | <u>5.67</u>          |
|                 |     |            |            |            | 5.6                  |

Table LXIX.

Exp. 2. KCl = 0.02 n. KOH originally  $2 \times 10^{-4}$  n.  $p_H$  at the end of dialysis (6 days) in inner liquid 5.68, in outer liquid 5.72, mean 5.70. Conductivity  $\kappa = 2.50 \times 10^{-3}$  and  $\kappa = 2.51 \times 10^{-3}$ , mean  $\kappa = 2.51 \times 10^{-3}$ . Migration anodic.

| $i \times 10^3$ | $t$ | $\Delta_L$ | $\Delta_R$ | $\Delta_M$ | $u_{20} \times 10^5$ |
|-----------------|-----|------------|------------|------------|----------------------|
| 2.020           | 120 | +4.56      | -4.56      | 4.56       | 5.99                 |
| 2.020           | 123 | +4.56      | -4.64      | 4.60       | 5.90                 |
| 2.020           | 123 | -4.56      | +4.64      | 4.60       | 5.90                 |
|                 |     |            |            |            | 5.9                  |

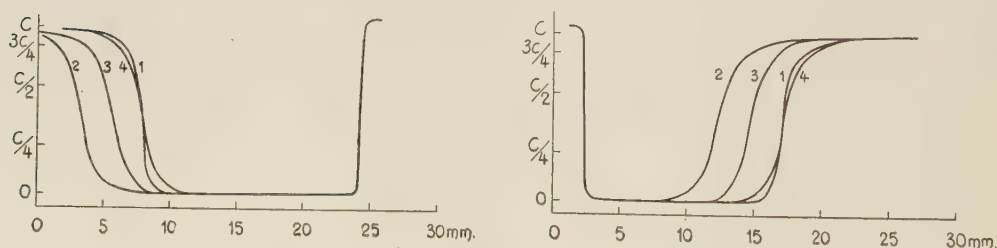


Fig. 33.

Fig. 33 shows the diagrams from exp. 1. The mobility  $u_{20} = 5.6 \times 10^{-5}$  obtained at  $p_H = 4.26$ , does not deviate much from the value in 0.02 n acetate buffer of the same  $p_H$  ( $u_{20} = 5.3 \times 10^{-5}$ ).

*R-Phycocerythrin.* For this protein the procedure followed in the phycocyan experiments could not be used since the available amount of protein was insufficient. The solutions were therefore prepared by dialysis against an outer liquid free from protein. The  $p_H$  of the outer liquid in the second experiment below is uncertain; else no troubles occurred in the  $p_H$  measurements.

Table LXX.

Exp. 1. KCl = 0.02 n. HCl originally  $10^{-3}$  n in inner liquid,  $2 \times 10^{-4}$  n in outer liquid.  $p_H$  at the end of dialysis (1 day) equal for both solutions:  $p_H = 3.47$ .  
 $\alpha = 2.59 \times 10^{-3}$  in both solutions.

| $i \times 10^3$ | $t$ | $A_L$ | $A_R$ | $A_M$ | $u_{20} \times 10^5$ |
|-----------------|-----|-------|-------|-------|----------------------|
| 2.040           | 120 | -8.94 | +8.16 | 8.55  | 11.48                |
| 2.040           | 120 | +8.26 | -8.58 | 8.42  | 11.31                |
| 2.040           | 129 | +9.46 | -9.22 | 9.84  | 11.67                |
|                 |     |       |       |       | 11.5                 |

Table LXXI.

Exp. 2. KCl = 0.02 n. Inner and outer liquids both free from acid or base in the beginning of dialysis.  $p_H$  at the end of dialysis (10 days) in inner liquid 4.68.  
 $\alpha = 2.52 \times 10^{-3}$  and  $2.50 \times 10^{-3}$ , mean  $2.51 \times 10^{-3}$ .

| $i \times 10^3$ | $t$ | $A_L$ | $A_R$ | $A_M$ | $u_{20} \times 10^5$ |
|-----------------|-----|-------|-------|-------|----------------------|
| 2.001           | 120 | +4.20 | -3.82 | 4.01  | 5.32                 |
| 2.001           | 120 | -4.12 | +4.62 | 4.37  | 5.80                 |
| 2.001           | 120 | +4.10 | -3.56 | 3.83  | <u>5.08</u>          |
|                 |     |       |       |       | 5.4                  |

The values agree fairly well with those obtained in 0.02 n sodium acetate buffers (fig. 22). This result, and the similar result obtained with phycocyan above indicates that differences between electrolyte media as regards protein electrophoresis become of importance only in case of a marked difference in valency type of the ions present, as in the barium acetate and phosphate-citrate buffer experiments described above.

## Discussion of the results of the measurements.

### The isoelectric points.

First it should be emphasized that the electrophoretic mobility of the proteins investigated is a well-defined, reproducible property of the substances, being sufficiently different to be used as a characteristic. As a means of distinguishing between different proteins in mixtures the determination of the mobility has the advantage of not being connected with any great change in the state of the solution, as are methods depending upon chemical changes, precipitation and so on. Some applications of electrophoresis in this direction will be found in the last chapter.

Earlier workers on electrophoresis very often report an electrophoretic movement towards both electrodes in the neighbourhood of the isoelectric point (73, 75). Attempts have even been made to explain this observation on the basis of the MICHAELIS theory of ampholytes, according to which a protein at its isoelectric point consists of the same amount of positive and negative particles (74). According to the discussion above (p. 26) this view is not correct. An electrophoretic migration towards both sides must always be taken as an indication of non-uniformity.

It is seen from the tables above that even in the immediate neighbourhood of the isoelectric point, the boundaries move with very nearly the same velocity in the same direction. Especially striking is the experiment 3 with hemocyanin (table XXXIII, p. 63).

It seems probable that the observation by other authors mentioned above depend upon heat convection, diffusion or non-uniformity of the material studied.

In most cases the electrophoretic mobility is an almost linear function of  $p_H$  in the interval  $\pm 0.5$  in  $p_H$  on both sides of the isoelectric point generally studied. Consequently, as has already been mentioned, the electrophoretic behaviour of a protein in this  $p_H$ -region may approximately be characterized by two constants: the isoelectric point  $p_H^\circ$  and the slope of the  $u$ - $p_H$  curve at the isoelectric point  $\left(\frac{du}{dp_H}\right)^\circ$ . The following table LXXII gives a survey of the results obtained in 0.02 N sodium acetate buffer solutions for the six proteins that were studied.



Table LXXII.

Isoelectric points and slopes of the isoelectric curves for different proteins in 0.02 n sodium acetate buffer.

| Protein                       | $p_H^\circ$ | $\left(\frac{du}{dp_H}\right)^\circ \times 10^5$ |
|-------------------------------|-------------|--------------------------------------------------|
| R-Phycoerythrin . . . . .     | 4.25        | 14.2                                             |
| Egg albumin . . . . .         | 4.55        | 10.4                                             |
| C-Phycocyan . . . . .         | 4.76        | 9.1                                              |
| Serum albumin . . . . .       | 4.88        | 9.1                                              |
| Helix Hemocyanin . . . . .    | 5.05        | 7.3                                              |
| Bence Jones protein . . . . . | 5.20        | 5.8                                              |

It is evident that the slope of the curve is smaller, the higher the isoelectric point is situated. For a certain protein, the slope  $\frac{du}{dp_H}$  generally increases towards the acid side.

The experiments in buffers of varying concentration (p. 58 and p. 74) and of varying composition (p. 67—71) prove that not only hydrogen ions but also other ions influence the mobility, the valency type and the concentration being of special importance.

Perhaps the most important result of measurements like these is that the isoelectric point is determined with comparatively great accuracy. However, it should be born in mind that the value obtained is valid only for the medium in question and that differences in the  $p_H^\circ$  value were observed in different media. The method is direct, if according to HARDY (33) the isoelectric point is defined as the acidity at which the substance is »isoelectric with the medium», and therefore does not migrate. This should be noted, since often measurements of acid-base combining capacity, coaguability and viscosity are stated as methods of determining the isoelectric point, the assumption being made that the maxima resp. minima of these properties would correspond to the point of zero mobility.

A similarly direct method would be to determine the isoelectric point from the zero point of membrane potentials. This method is applicable especially to non-buffered solutions, of no or low salt content, whereas the electrophoretic method is of special value in buffered solutions and solutions of comparatively high salt content.

As mentioned above (p. 10) qualitative electrophoresis experiments have been made by a great many workers, with the purpose of finding the isoelectric point. Of the proteins studied in this work, R-phycoerythrin, C-phycocyan, and Bence Jones protein have not been investigated before, as far as is known to the author.

Egg albumin was investigated recently by ITO and PAULI (75). They found  $p_H^\circ = 4.60$  (in 0.005 n acetate buffer), in good agreement with the value 4.55 obtained in this work. They used non-crystallized, electro-dialyzed material.

Serum albumin was first studied by MICHAELIS and DAVIDSOHN (76), who found  $p_H^\circ = 4.7$  in 0.1 n and 0.02 n acetate buffer. ITO and PAULI (75) found  $p_H^\circ = 4.99$  in 0.005 n acetate buffer.

Helix hemocyanin was investigated by STEDMAN and STEDMAN (77), who found  $p_H^\circ =$  about 5.3 for dialyzed Helix blood, mixed with acetate buffer.

Qualitative experiments have also been made by different workers, using neutral salts as media, but judging from the experiment p. 28 with egg albumin in potassium chloride solution, the results obtained must be doubtful on account of the insufficient buffering action of the medium which gives rise to boundary anomalies.

There are a number of other properties of proteins that vary in a characteristic manner with the acidity of the medium in which the protein is dissolved, and that are connected with the charge of the protein. Membrane potentials and the osmotic pressure should be mentioned first of these. Both are closely related to the charge according to the DONNAN theory of membrane equilibria (see p. 32). In fact, measurements of membrane potentials have been utilized for the determination of the charge of colloidal particles by BJERRUM (47) and by RINDE (48). It was mentioned above that it should be possible to determine also the isoelectric point in this way. There are as yet very few measurements on membrane equilibria in nearly isoelectric protein solutions, the only available data being those of LOEB (46) and of ADAIR (78). For gelatin LOEB was able to confirm DONNAN's theory and found the minimum of osmotic pressure at  $p_H = 4.7$ . Also for egg albumin he found the minimum of osmotic pressure and the zero membrane potential at  $p_H = 4.7$ .

ADAIER has made a very extensive and careful study of osmotic pressure and membrane equilibria in hemoglobin solutions. Among other things also the  $p_H$ -variation of these properties was studied. He found that, in agreement with DONNAN's theory, the osmotic pressure in a buffered medium was almost independent of the acidity over a  $p_H$ -range of about 5 to 9. In absence of salts (only small amounts of ammonia or carbon dioxide were present) an evident minimum was obtained at  $p_H = 6.8$ , which is in agreement with the electrophoretic isoelectric point  $p_H^\circ = 6.8$  determined by MICHAELIS and collaborators (36 c, g).

The charge of proteins also influences their behaviour in the SVEDBERG ultra-centrifuge. SVEDBERG (50) worked out a theory of sedimentation, diffusion and sedimentation equilibrium of colloid electrolytes in analogy to the NERNST diffusion theory of electrolytes. For the sedimentation equilibrium the author (49) extended this theory to colloids only partially combined with ions, and by a thermodynamical treatment could show the analogy of these charge effects to those found in the DONNAN equilibrium. According to this theory, there is a close correspondence

between the osmotic pressure and the concentration distribution in the sedimentation equilibrium with respect to the »DONNAN effects» — both methods give too low values of the molecular weight except at the isoelectric point. The author also pointed out that by addition of a sufficient quantity of a neutral salt these effects can be depressed, not only in the sedimentation equilibrium but also in sedimentation and diffusion, and the true molecular weight be obtained.

It is a consequence of these considerations that in solutions of a protein, free from salts, and containing a small quantity of acid or base, a maximum value of the apparent molecular weight should be obtained at the isoelectric point. Most determinations with the ultracentrifuge have been made in buffered media to eliminate such effects. Only some (unpublished) measurements of the sedimentation velocity of egg albumin by NICHOLS, performed in dilute HCl and NaOH, can be used for the purpose in question. He obtained an evident maximum at  $p_H = 4.65$ .

Another property that has been used for the determination of the isoelectric point is the acid-base combining capacity, but, contrary to the effects previously related, the application of this property for the purpose in question involves the assumption that only hydrogen resp. hydroxyl ions are responsible for the charge.

Numerous investigations have been made, mainly with the potentiometric method.

A good survey of the results is given by COHN (79). However, comparatively few measurements have been made in the neighbourhood of the isoelectric points where also, of course, the experimental difficulties are greater. The values of different workers vary a great deal in the position of the zero point, but the agreement in the shape of the curves obtained is better. This is natural, since even very small traces of acid or base present as impurity may change the position of the curve considerably, the combining capacity being as low as  $2-3 \times 10^{-4}$  equivalents per gram of protein at a  $p_H$  distance of 1.0 from the zero point.

According to LOEB (46) the acid combining capacity is the same for different acids. For weak acids, however, the calculation of the combining capacity is very uncertain.

Very extensive measurements have been made on crystallized egg albumin by SØRENSEN (80) and by LINDERSTØM-LANG and LUND (81). They found the zero point (»isoionic reaction» according to LINDERSTØM-LANG) at  $p_H = 4.80$  in sulfuric acid-ammonium sulphate mixtures and at  $p_H = 4.88$  in hydrochloric acid-ammonium chloride mixtures. According to LINDERSTØM-LANG the difference between these two values may, partially at least, be due to diffusion potentials.

These values deviate markedly from the value obtained by electrophoresis in acetate buffer ( $p_H^\circ = 4.55$ ). Also the experiment with egg albumin (p. 28) shows that at  $p_H = 4.81$ , in KCl, this protein carries negative charge.

Determinations of acid combining capacity in buffered solutions are difficult on account of the small  $p_H$ -changes that have to be determined. However, an



attempt was made to determine the isoionic reaction in 0.02 n acetate buffer for the egg albumin used in the experiments above. To obtain measurable  $p_H$ -changes it proved necessary to use a protein concentration much higher than in the electrophoresis experiments. Buffer solutions of varying  $p_H$  and constant acetate concentration 0.04 n were diluted with an equal volume of water (free of carbon dioxide) or of a 5.13 % electrodialyzed egg albumin solution taken directly out of the electrodialysis apparatus. The  $p_H$  of the solutions were measured. The results are given in the following table.

*Table LXXIII.*

Changes in  $p_H$  produced in 0.02 n acetate buffers by the presence of 2.56 % egg albumin.

| Sodium acetate conc. | Acetic acid conc. | Buffer potential volts | Protein potential volts | Protein $p_H$ | Difference volts |
|----------------------|-------------------|------------------------|-------------------------|---------------|------------------|
| 0.02 n               | 0.008 n           | 0.6310                 | 0.6256                  | 4.98          | + 0.0054         |
| 0.02 n               | 0.012 n           | 0.6205                 | 0.6186                  | 4.86          | + 0.0019         |
| 0.02 n               | 0.020 n           | 0.6078                 | 0.6091                  | 4.70          | - 0.0013         |
| 0.02 n               | 0.028 n           | 0.5991                 | 0.6014                  | 4.57          | - 0.0023         |

The minimum point (isoionic reaction) is evidently situated at  $p_H = 4.76$ .

It seems therefore that for egg albumin there really is a difference between isoelectric and isoionic reaction, and that the charge of this protein is not determined by the hydrogen and hydroxyl ions solely. Other ions must be assumed to be able to combine with the protein too. This assumption is in agreement with the conclusion arrived at by several workers (8) that proteins are able to combine with neutral salts (which also has been proved to be the case for amino acids). It is, however, necessary also to assume that egg albumin has a greater affinity towards anions, even at  $p_H$  values where it is negatively charged. Thus, at  $p_H = 4.76$ , in sodium acetate buffer, the negative charge of egg albumin corresponding to the mobility  $2 \times 10^{-5}$  cm/sec. would be due to bound acetate ions only, and at  $p_H = 4.55$  (the isoelectric point), the amount of hydrogen ions bound would be equal to the surplus amount of bound acetate ions (i.e. bound acetate ions minus bound sodium ions). The latter amount may therefore be calculated from the known acid combining capacity of egg albumin (about  $20 \times 10^{-5}$  eq. per gram protein and  $p_H$  unit); it becomes  $(4.76 - 4.55) \times 20 \times 10^{-5} = 4.2 \times 10^{-5}$  eq. per gram at the isoelectric point.

This view is strongly supported by some results arrived at by ADAIR (78) in the above mentioned work on osmotic equilibria with hemoglobin. By using very high protein concentrations (17—20 % or even more) he was able to study accurately the distribution of different ions between protein solution and outer liquid in equilibrium quite in the neighbourhood of the isoelectric point. ADAIR found that



there was a large excess of ions inside the membrane and that the amount of anions much exceeded that of cations, both facts in contradiction to the simple DONNAN theory. He concluded that the »true» isoelectric point (the point of zero charge) is distinctly on the acid side of the point of zero acid-base combining capacity.

The greater affinity for anions was evident even at a  $p_H$  value of 7.8, where hemoglobin carries a strongly negative charge ( $p_H^\circ = 6.8$ ).

In any case, we must of course assume, that the variation of electrophoretic mobility in a medium of varying  $p_H$  but with all other ions of constant concentrations such as the acetate buffer solutions used is mainly due to a variation of the amount of hydrogen ions bound. It should be observed that the acid-base combining capacity curve is mostly an approximately straight line in the neighbourhood of the isoelectric point, like the mobility curves obtained above.

LINDERSTRØM-LANG showed that a straight line should be obtained for the acid combining capacity if we assume that each hydrogen ion, in the neighbourhood of the isoelectric point, is bound to the protein with an affinity that does not vary with the amount of hydrogen ions bound (81).

Some other properties are generally assumed to be connected with the charge of the protein even if it has not been possible to formulate this relationship quantitatively.

The variation of protein solubility with  $p_H$  is generally explained on the basis of the MICHAELIS theory of ampholytes (82), according to which the solubility of an ampholyte varies in proportion to its electrolytic dissociation. Consequently, the isoelectric point, where the neutral molecules have the maximum concentration, is the point of minimum solubility. This was confirmed by MICHAELIS and DAVIDSON (83) for the  $p_H$ -variation in solubility of m- and p-amino-benzoic acid, and asparagic acid. MICHAELIS extended his theory to include proteins too: the variation of the properties of proteins with the acidity (especially the variation of solubility), should be in analogy to the behaviour of simple amino acids. In fact, close agreement was found between the isoelectric point determined by electrophoresis and the solubility minimum: For serum globulin  $p_H^\circ = 5.4$  and the point of maximum precipitation by dilution  $p_H = 5.42$  (84), for casein  $p_H^\circ = 4.6$ , precipitation maximum  $p_H = 4.62$  (85).

For the proteins studied in this work the variation of solubility with  $p_H$  has been studied only for phycoerythrin and egg albumin. LEMBERG (59 c) found for R-phycoerythrin a precipitation optimum between  $p_H = 4.25$  and  $4.34$  in sodium acetate buffers in good agreement with the isoelectric point. ( $p_H^\circ = 4.25$ , p. 62 above.)

SØRENSEN and HØYRUP (86) studied the variation of solubility with  $p_H$  for egg albumin in ammonium sulphate solutions. They found a pronounced minimum at  $p_H = 4.58$ . This is very near to the isoelectric point determined by electrophoresis ( $p_H^\circ = 4.55$ ). The media are, however, different and therefore we can hardly draw any definite conclusions from this agreement.

### The mobility and the charge.

According to SVEDBERG (87) the molar frictional constant  $f$  for a protein may be calculated from its molecular weight  $M$  (determined by the sedimentation equilibrium method) and its sedimentation constant  $s$  (determined by sedimentation velocity measurements) from the expression

$$f = \frac{M(1 - V\rho)}{s}, \quad (46)$$

if  $V$  is the partial specific volume of the protein and  $\rho$  the density of the medium. From the values of the mobilities  $u$  obtained in the above measurements we may calculate the charge  $E_a$  (electrostatic units) per mol of protein from the equation

$$E_a = f \times u \quad (47)$$

or, since  $u$  was expressed in  $\text{cm}^2 \text{ sec}^{-1} \text{ volt}^{-1}$

$$E_a = 300 \times f \times u \text{ (e.s.u. per mol)}. \quad (48)$$

By dividing the expression with  $96,494 \times 3 \times 10^9$  (the charge of one gramequivalent in e.s.u.) we obtain the charge in elementary charges per particle, that is, the mean valency of the protein particle.

As will be discussed below  $E_a$  does not represent the actual charge of the particle and will therefore be called the apparent charge.

In the following table the magnitude of the apparent charge in the isoelectric region in 0.02 *n* acetate buffers for the proteins studied above has been calculated. The molar frictional coefficients have been taken from table VII in the work of SVEDBERG (87). For the sake of simplicity use has been made of the values of  $\frac{du}{dp_H}$  in the isoelectric region obtained above (see table LXXII), and the value  $\frac{dE_a}{dp_H}$  has been calculated in each case. Since  $\frac{du}{dp_H}$  was found to be approximately a constant near the isoelectric point the apparent charge  $E_a$  in this region is obtained from the expression

$$E_a = (p_H^\circ - p_H) \frac{dE_a}{dp_H}. \quad (49)$$

Table LXXIV.

| Substance           | Mobility per $p_H$<br>unit in the iso-<br>electric region<br>$\left(\frac{d u}{d p_H}\right)^5$ | Molar<br>frictional<br>coefficient<br>$f$ | Apparent charge<br>per mol and $p_H$<br>unit<br>$\frac{d E_a}{d p_H}$ (e.s.u.) | Apparent charge<br>per $p_H$ unit, in<br>elementary char-<br>ges per particle.<br>(Valency per $p_H$<br>unit.) |
|---------------------|-------------------------------------------------------------------------------------------------|-------------------------------------------|--------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------|
| R-Phycoerythrin     | $14.2 \times 10^{-5}$                                                                           | $4.61 \times 10^{16}$                     | $19.6 \times 10^{14}$                                                          | 6.8                                                                                                            |
| Egg albumin         | $10.4 \times 10^{-5}$                                                                           | $2.63 \times 10^{16}$                     | $8.2 \times 10^{14}$                                                           | 2.8                                                                                                            |
| C-Phycocyan         | $9.1 \times 10^{-5}$                                                                            | $4.74 \times 10^{16}$                     | $12.9 \times 10^{14}$                                                          | 4.5                                                                                                            |
| Serum albumin       | $9.1 \times 10^{-5}$                                                                            | $4.01 \times 10^{16}$                     | $11.0 \times 10^{14}$                                                          | 3.8                                                                                                            |
| Helix Hemocyanin    | $7.3 \times 10^{-5}$                                                                            | $13.50 \times 10^{16}$                    | $29.6 \times 10^{14}$                                                          | 10.2                                                                                                           |
| Bence Jones protein | $5.8 \times 10^{-5}$                                                                            | $2.48 \times 10^{16}$                     | $4.3 \times 10^{14}$                                                           | 1.5                                                                                                            |

The assumption underlying eq. (47) that the force acting upon the particle is the product of the particle charge and the potential gradient is not correct. The potential of a particle in a medium containing free ions is always to some extent reduced by the action of the surrounding ionic atmosphere. This question was first treated by GOUY (88) and independently by CHAPMAN (89) and the problem became of a very general importance for the whole electrochemistry when DEBYE and HÜCKEL (90) showed that the electrostatic interaction between ions in electrolyte solutions may, at least in the first approximation, be treated in an analogous way. HÜCKEL (91) also applied the theory to the problem of electrophoresis.

As regards the development and the present state of the theories of electrophoresis it may be sufficient here to refer to the survey and literature given in the recent work of PAULI and VALKÓ (8). For the present discussion it is of special interest that HÜCKEL could show that, at least for spherical particles, electrophoresis and free ionic migration are related phenomena, both being described by the equation

$$u = \frac{K \zeta}{6 \pi \eta} \quad (50)$$

Here  $u$  is the mobility,  $K$  the dielectric constant,  $\zeta$  the potential of the particle and  $\eta$  the viscosity of the medium.

If the charge of the particle is  $E$  and the radius  $r$ , the potential in a medium free of ions is

$$\zeta = \frac{E}{K r} \quad (51)$$

and eq. (50) becomes

$$u = \frac{E}{6 \pi \eta r} \quad (52)$$

which evidently is identical with eq. (47) if we assume that STOKES' law is valid.

In a medium containing ions a »diffuse double layer» is formed around the particle and eq. (51) becomes

$$\zeta = \frac{E}{K r} \times \frac{d}{d+r} \quad (53)$$

where  $d$  is the thickness of the double layer, a quantity identical with the corresponding function  $1/\kappa$  in the DEBYE-HÜCKEL theory of strong electrolytes (90). It may consequently be calculated from the following equation (monovalent ions)

$$d = \frac{1}{\kappa} \approx \frac{3 \times 10^{-8}}{\sqrt{\gamma}} \text{ (cm.)} \quad (54)$$

at least for the low potential values in the neighbourhood of the isoelectric point. (Here  $\gamma$  is the concentration in mol per litre.)

In 0.02 N sodium acetate we thus find  $d \approx 2 \times 10^{-7}$  cm. We know from the ultracentrifugal determinations that the particles of the proteins in table LXXIV are all spherical, with exception for serum albumin. Therefore we may apply eq. (50). The particle radius of egg albumin is  $2.2 \times 10^{-7}$  cm. (87), consequently nearly equal to the double layer thickness  $d$ . The actual charge of the particle would therefore be about twice the apparent charge.

The DEBYE-HÜCKEL theory of electrophoresis has not yet been subject to any thorough experimental test. One consequence of the theory, viz. the influence of the shape of the particle upon the mobility, is not in agreement with the experimental facts, as has been shown recently in an investigation by ABRAMSON (92).

Also it is questionable if the comparatively small charge on the particles (see table LXXIV) may be regarded as evenly distributed over the whole surface, an assumption underlying the equations (51, 53 and 54). If the charge is carried by bound ions one would rather believe that it is localized to the regions of the surface where these ions are situated.

The value of the actual charge arrived at by the above calculation must therefore be regarded as very uncertain.

On the other hand the success with which the DEBYE-HÜCKEL theory has been applied to solutions of ordinary electrolytes and the consequence of the theory that the influence of the double layer must become of greater importance with increasing particle radius makes it very probable that effects of the kind



discussed play an important rôle in protein solutions, and that the apparent charge obtained from calculations like those above is considerably smaller than the actual charge.

Other methods of determining the charge of colloidal particles (e.g. DONNAN potential measurements, acid-base combining capacity determinations) must be assumed to be subject to a similar influence of the electrolyte medium. The result obtained in electrolyte media must therefore be considered as an apparent charge.

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## The electrophoresis of mixtures and of non-uniform proteins.

In mixtures colloids do not generally retain their properties unchanged, at least not if they carry opposite charge. According to BILLITER (93) a  $\text{Fe}_2\text{O}_3$  sol (positive) precipitates an  $\text{As}_2\text{S}_3$  sol (negative) within certain ranges of concentration. In concentration regions where precipitation does not occur the electrophoresis is influenced strongly.

Another phenomenon of this kind is the protective action exerted by certain colloids towards electrolyte precipitation of hydrophobic sols. This effect, however, does not seem to depend upon the charges of the two substances.

Mutual precipitation is generally only observed in cases when at least one of the colloids mixed can easily be coagulated by electrolytes. Colloids of the hydrophilic type, like proteins, seem to behave differently when they are mixed.

Thus it was found that when solutions of 0.2% phycoerythrin and 0.2% Bence Jones protein in acetate buffers of  $p_H = 4.67$  were mixed in different proportions no precipitation took place, even though at this  $p_H$  the two proteins carry opposite charge. ( $p_H^\circ = 4.25$  and  $5.20$ ). Also it was found that a hemoglobin solution did not precipitate a phycoerythrin solution in a phosphate buffer of  $p_H = 5.50$ , although the difference in charge was still more pronounced ( $p_H^\circ = 4.25$  and about  $6.7$ ).

That precipitation in some cases may still occur when the differences in charge are great is evident from some observations made by BETH AF UGGLAS (94). She found that the proteins histon and protamin give precipitates with hemoglobin and casein. The I.P. of the former proteins are about  $p_H^\circ = 8.5$  and  $p_H^\circ = 12$  (95).

The fact that in most cases no precipitation occurs does not, of course, prove that the substances mixed do not influence each other at all. The charge can not be the only factor determining the solubility of proteins since many proteins are soluble at their isoelectric points.

Till recently our views of the chemical individuality of native high molecular substances in solution were almost entirely based upon the results of the application of fractional precipitation methods. The classical method of separating and characterizing proteins by means of precipitation with ammonium sulphate is perhaps the best example of such methods. Against this method may be raised several objections. The precipitation process means a rather violent change of the state of the solution that may be supposed to bring about irreversible changes of the sub-

stances precipitated, as in the case of the coagulation of colloids by electrolytes. In fact, it has often been found that part of the precipitate formed cannot be brought into solution again. Also the precipitates probably adsorb certain amounts of other substances present in the solution. Conclusions drawn from the composition of the precipitate, as regards the composition of the chemical individuals existing in solution, must therefore be regarded as uncertain.

Our conceptions of chemical individuality are based upon our methods of separation. Therefore, for the study of substances as unstable as most biocolloids, the development of separation methods that do not involve great changes in the conditions of existence of these substances must be of great value.

It has already been pointed out above (p. 9) that the study of electrophoresis by the moving boundary method forms a possibility of observing the separation of differently migrating proteins in certain cases provided that they do not influence each other's migration too much. This method, and the centrifugal methods used for the same purpose by SVEDBERG and his collaborators (especially LAMM) (23) are the least violent methods of separation hitherto applied for the purpose of studying the chemical individuality of proteins.

To give an idea of the applicability of the method used in the present work for this purpose, some experiments concerning the behaviour of some artificial mixtures in electrophoresis will first be described.

### Electrophoresis of artificial mixtures.

An experiment was first made to decide whether the phycoerythrin in the mixture with Bence Jones protein discussed in the preceding section showed the same mobility as in a pure buffer medium. Long-wave ultraviolet was used, since in this region the Bence Jones protein does not absorb, and only the movement of phycoerythrin is therefore obtained. In the right tube the phycoerythrin boundary was allowed to migrate downwards a considerable distance, the B.J. protein boundary migrated upwards, and consequently, in this tube, the phycoerythrin migrated in a medium of B.J. protein of almost constant concentration. At the same time, in the other tube, the phycoerythrin migrated in pure buffer, free from protein. The mobilities were calculated for each boundary separately (table LXXV).

Table LXXV.

0.2 % phycoerythrin and 0.2 % Bence Jones protein. Sodium acetate 0.02 n. Acetic acid 0.02 n.  $p_H = 4.67$ . Migration anodic.

| $i \times 10^3$ | $t$ | $\Delta_L$ | $\Delta_R$ | $u_L \times 10^5$ | $u_R \times 10^5$ |
|-----------------|-----|------------|------------|-------------------|-------------------|
| 1.524           | 180 | +8.14      | -7.44      | 5.42              | 4.96              |
| 1.524           | 50  | +2.14      | -1.94      | <u>5.13</u>       | 4.65              |
|                 |     |            |            | 5.28              | 4.81              |

The mobility therefore seems to be decreased somewhat by the presence of the other protein. (The  $u_L$  value fits well into the  $p_H$ -mobility curve for phycoerythrin p. 62.)

We can draw the conclusion that oppositely charged proteins can exist together in solution without influencing each other to any great extent. The electrostatic interaction between the particles is evidently not as great as between two colloids like  $As_2S_3$  and  $Fe_2O_3$ . Proteins seem, in this respect, to be more similar to ordinary electrolytes.

An experiment in which were measured the mobilities of two proteins in a mixture, both with the same charge, will be described below (p. 91).

If photographs of the electrophoresis of protein mixtures are taken in the short-wave ultraviolet, we obtain diagrams corresponding to the superimposed diagrams of each separate component's migration. If there was no diffusion, and if the boundaries at start were completely sharp we would expect to be able to observe each boundary separately. In reality, however, this is possible only for substances of widely differing mobilities. In other cases the separation is insuf-

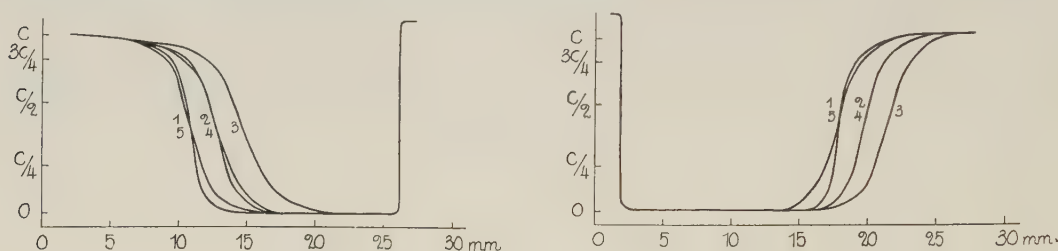


Fig. 34.

ficient to give diagrams with separate boundaries, even if the deformation of the curves clearly shows that the migration is not uniform.

Examples of such experiments are given below. Fig. 34 shows the diagrams from an experiment with a mixture of 0.2% egg albumin and 0.1% phycocyan in 0.02 n acetate buffer of  $p_H = 4.97$ . The difference in mobility is  $2.4 \times 10^{-5}$ . The current intensity was  $1.176-1.180 \times 10^{-3}$ , and the time interval between two successive exposures 120 minutes. After the third exposure the current was reversed.

The curves look in this case almost like those of a single uniform protein. However, especially in the left diagram (left tube) an abnormal behaviour is clearly visible: the slope of the curves decreases during the first two time intervals, after which it increases again. This »reversible» blurring of the boundaries is characteristic of the electrophoresis of mixtures and is of course due to the separation of differently migrating components, which process is reversed when the direction of the current is changed.

A better separation could have been obtained by using stronger current. This would have been quite feasible, for since the effect was only 1.7 watt/cm<sup>2</sup>, current at least twice as strong might have been used without any risk for heat convections (p. 40).



More marked effects are demonstrated in fig. 35. This is an experiment with a mixture of 0.2 % egg albumin and 0.2 % Bence Jones protein in a 0.02 n acetate buffer of  $p_H = 4.67$ . The difference in mobility is  $4.5 \times 10^{-5}$ . The current intensity was taken high:  $2.654 \times 10^{-3}$  amp., the time intervals 60 minutes each. The current had all the time the same direction. Here the non-uniformity of the migration is clearly visible, but it is hardly possible to calculate the migration velocities of the components.

Fig. 36 shows the curves from an experiment with a mixture of 0.1 % phycoerythrin with 0.1 % phycocyan, in a 0.01 m phosphate buffer 1:1 of  $p_H = 6.94$ . The current intensity was  $1.150 \times 10^{-3}$  amp., the time intervals 40 min. The direction

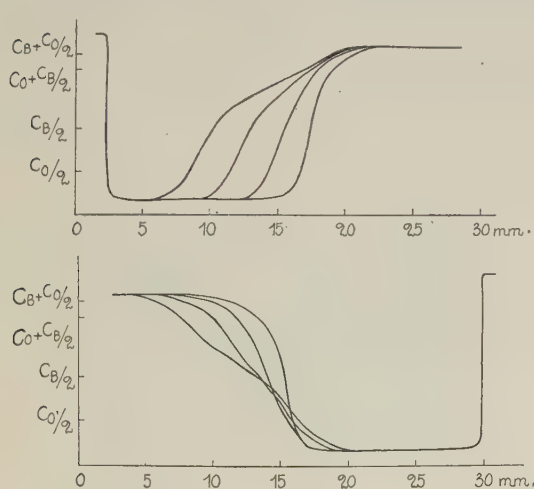


Fig. 35.

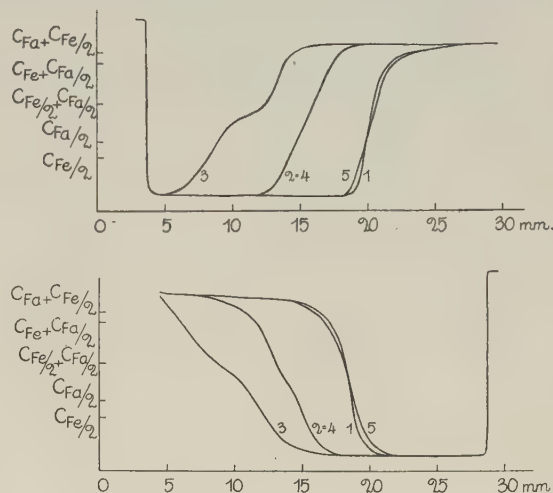


Fig. 36.

of current was reversed after the third exposure. The concentration exposures were not taken as usual but with the mixtures marked out in the diagram.

The separation is sufficient to justify an attempt to calculate the mobilities, at least for the interval 1—3, by measuring the distances of the point  $C_{Fe/2} + C_{Fa/2}$  in curve 1 to the points  $C_{Fe/2}$  and  $C_{Fe} + C_{Fa/2}$  in curve 3 of the upper diagram and similarly the distances  $C_{Fe/2} + C_{Fa/2}$  to  $C_{Fa/2}$  and  $C_{Fa} + C_{Fe/2}$  in the lower diagram, curves 1 and 3.

We obtain for the phycoerythrin 12.60 mm. in the right tube, 12.80 mm. in the left, for phycocyan 6.78 mm. and 6.84 mm. respectively. This gives the mobilities  $21.7 \times 10^{-5}$  for phycoerythrin and  $11.7 \times 10^{-5}$  for phycocyan. Both values agree fairly well with those found in the experiments p. 73 and 75.

The curves 4 and 5 in the diagrams of this experiment demonstrate the reversibility of the effects. The differences between curves 2 and 4 are hardly noticeable on the record plates. The differences between these and between 1 and 5 should be due to diffusion only.

It should be observed that the boundary anomalies also give rise to effects that are reversed, when the direction of the current is changed.

Experiments for the investigation of uniformity of migration must therefore be made in media, where such anomalies have been eliminated, as was the case in the above experiments. The boundary anomalies are, however, easily distinguished from the effects due to inhomogeneous migration: the former give rise to contrary effects at the two boundaries (p. 29), whereas the latter produce either blurring of both or sharpening of both.

If it is a question of obtaining the greatest possible separation, the voltage on the apparatus must be increased. If the buffer solutions are diluted, very high

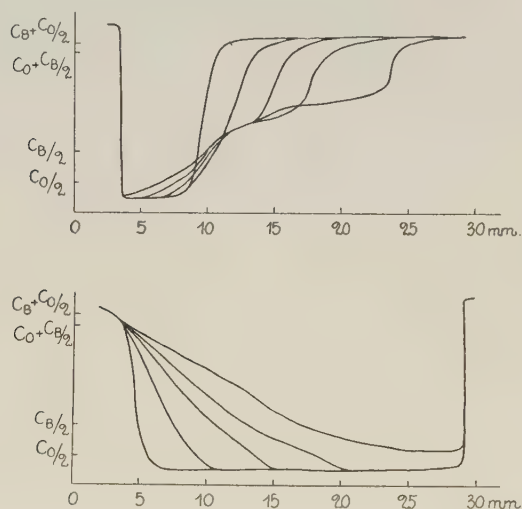


Fig. 37.

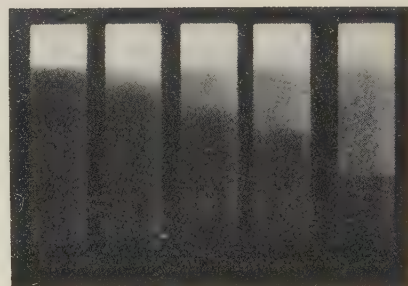


Fig. 38.

voltage can be used, but on account of the poor buffering action of the medium, boundary anomalies occur. Therefore, one boundary is completely blurred, whereas the other is sharpened. An example is given in fig. 37. It represents an experiment with the same mixture of egg albumin and Bence Jones protein that was used above (fig. 35), the buffer concentration, however, was only 0.004 n, the voltage applied about 5 times greater.  $p_H$  was 4.72, and  $\kappa = 3.20 \times 10^{-4}$ . The time intervals were 30 minutes, except for 4—5 that was 60 minutes. The current intensity was  $1.264\text{--}1.269 \times 10^{-3}$  amp., with the current closed in the same direction during the whole experiment. The heat effect was 8.7 watt/cm<sup>3</sup>.

The photographs of the right tube are shown in fig. 38.

Such experiments may be of value in cases when it is desired to find out whether a definite chemical individual is present as component in a mixture or not.

### The electrophoresis of native mixtures and of non-uniform proteins.

In some electrophoresis experiments with proteins that were believed at first to be uniform, curves similar to those in fig. 34—36 have been obtained, indicating that we have to deal with mixtures. Since a control of the chemical uniformity is perhaps the most important condition for successful work with high molecular substances, this application of electrophoresis is of considerable interest. Therefore some examples will be mentioned.

Fig. 39 shows the curves from an experiment with a sample of leukosin in 0.02 *n* acetate buffer of  $p_H = 4.16$ . The current intensity was  $1.650\text{--}1.660 \times 10^{-3}$  amp. the time intervals 120 minutes. The migration was cathodical. It is evident that the material is extremely non-uniform.

An investigation in the ultracentrifuge by B. SJÖGREN also indicated non-uniformity in the molecular weight, the apparent diffusion constant being ab-

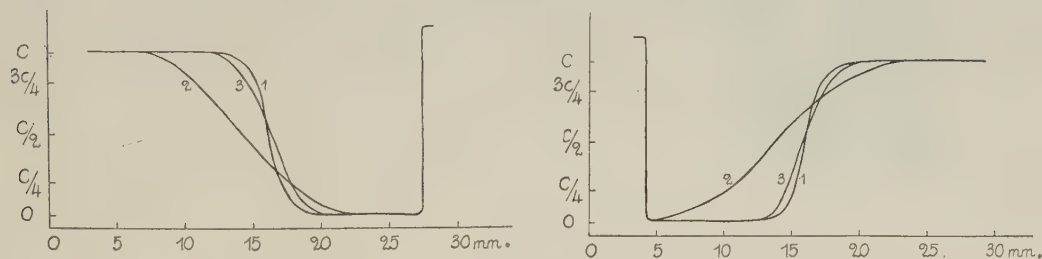


Fig. 39.

normally great and changing with time (from  $18 \times 10^{-7}$  to  $30 \times 10^{-7}$   $\text{cm}^2 \text{sec}^{-1}$  during the run).

Samples of fibrinogen and lactalbumin also showed marked non-uniformity in electrophoresis as well as in the sedimentation.

As mentioned above (p. 60), it was difficult to obtain serum albumin as uniform as the other proteins which were investigated. It was necessary to use quite freshly prepared solutions. In one case a sample was investigated, about 3 months old and was found non-uniform. One month later it was investigated in the ultracentrifuge and was found to contain about 20 % non-centrifugible material.

If the purification is insufficient, this may show up in the electrophoresis. It was mentioned above (p. 50) that phycocyan, that had been reprecipitated 3 times still contained a considerable amount of a foreign substance, showing light absorption in the long-wave ultraviolet and in the short-wave regions. Fig. 40 shows a photograph in the long-wave ultraviolet and fig. 41 gives the curves from an experiment with this material. The protein concentration was 0.18 %, the buffer was 0.02 *n* acetate buffer of  $p_H = 4.27$ . The time intervals were 1—2 60 min., 2—3 60 min., 3—4 210 min. The current intensity was  $1.465\text{--}1.480 \times 10^{-3}$  amp. The current was reversed after the third exposure.

The phycocyan may be sufficiently purified from this substance by crystallization to show uniform migration. This is demonstrated by fig. 42, corresponding to the experiment no. 2 p. 64, which was performed at  $p_H = 4.34$ .

Another example of insufficient purification is shown in fig. 43, giving the curves from an experiment with egg-white. The fresh egg-white had been whipped



Fig. 40.

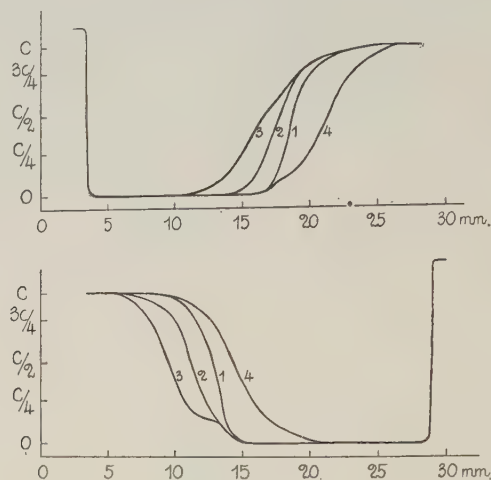


Fig. 41.

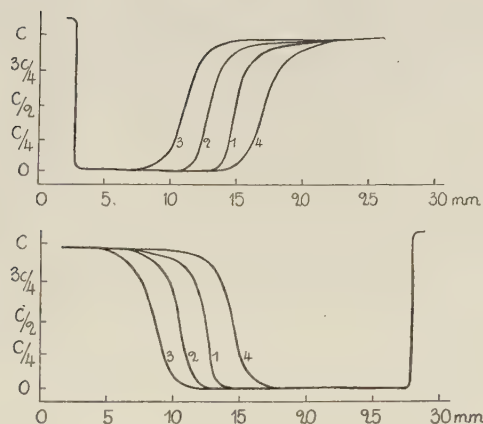


Fig. 42.

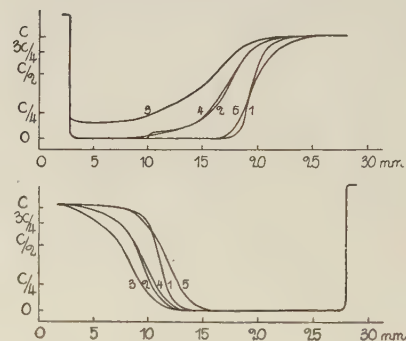


Fig. 43.

and then dialyzed for four weeks against distilled water. A globulin precipitate appeared after a few days. The dialyzed solution was filtered. Its conductivity was  $3.1 \times 10^{-4}$  at  $20^\circ.0$  and its protein concentration 7.63 %. A sample of this solution was investigated in 0.02 n acetate buffer of  $p_H = 4.35$ . The migration was cathodical. The time intervals were 60 min., the current intensity  $1.242\text{--}1.250 \times 10^{-3}$  amp. The current was reversed after the third exposure.



It is seen that the migration is strongly non-uniform and that a substance moving in the same direction but with greater mobility is present (probably ovomucoid).

An experiment at  $p_H = 4.78$  with the same material gave similar results as regards the uniformity.

A material that shows lack of uniformity in the ultracentrifuge does not always also give markedly non-uniform electrophoresis. An example of special interest is the reversible splitting up of protein molecules at  $p_H$ -values far from the isoelectric point. For example C-phyococyan, according to SVEDBERG and KATSURAI (56) at  $p_H = 6.8$  gives non-uniform sedimentation, corresponding to a mixture of 65 % with molecular weight 208,000 and 35 % with 104,000. The dissociation is reversibly changed with  $p_H$ ; at the isoelectric point the substance is uniform. The phycoerythrin molecule is decomposed first at higher  $p_H$  values. In an experiment in sodium-phosphate — sodium hydroxide buffer of  $p_H = 11.0$ . SVEDBERG and KATSURAI

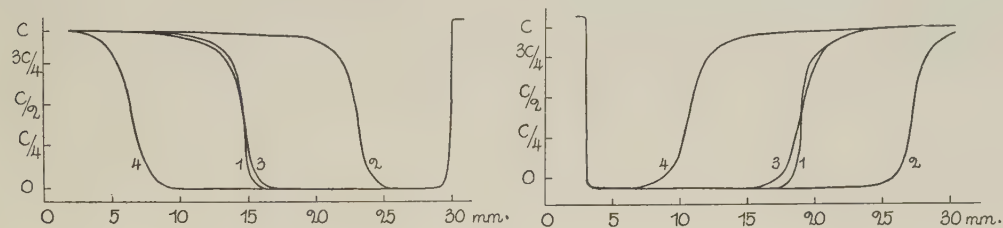


Fig. 44.

were able to show that the phycoerythrin consisted of about 75 % of molecular weight 208,000 and 25 % 34,600.

The electrophoresis experiments performed under the same conditions did not show any indication of non-uniformity at all. An experiment with phycocyan in phosphate buffer of  $p_H = 6.94$  has already been described above (p. 75). The migration was as uniform as in the neighbourhood of the isoelectric point. Fig. 44 gives the curves from an experiment with phycoerythrin at  $p_H = 10.95$ , where the differences should be still more marked. No trace of non-uniformity can be observed. The mobility was calculated (table LXXVI).

Table LXXVI.

0.2 % phycoerythrin in phosphate buffer of  $p_H = 10.95$ .

| $i \times 10^3$ | $t$ | $A_L$ | $A_R$ | $A_M$ | $u_{20} \times 10^5$ |
|-----------------|-----|-------|-------|-------|----------------------|
| 1.660           | 50  | +8.28 | -8.24 | 8.26  | 22.23                |
| 1.660           | 50  | -8.28 | +8.22 | 8.25  | 22.20                |
| 1.660           | 50  | -8.34 | +8.24 | 8.29  | <u>22.31</u>         |
|                 |     |       |       |       | 22.25                |

The conductivity was  $1.76 \times 10^{-3}$ . Long wave ultraviolet light was used.

Evidently the particle size, at least at these high mobilities, is insignificant for the magnitude of the electrophoretic mobility.

There may also be cases when a material, which with the ultracentrifuge has been found uniform as regards molecular weight, in electrophoresis is found non-uniform. The mixture of egg albumin and Bence Jones protein studied above would not give any separation at all in sedimentation, since these proteins have the same molecular weight. This case cannot be considered as unique. Very astonishing regularities in protein molecular weights have been found by SVEDBERG and his collaborators. All proteins investigated by them (except the very highmolecular hemocyanins) have molecular weights of approximately 34,500,  $2 \times 34,500$ ,  $3 \times 34,500$ , and  $6 \times 34,500$ . Proteins that are widely different in chemical composition and electrochemical properties may still have the same molecular weight.

It should also be observed that the electrophoretic mobility, being a surface property, may be influenced by a change in the chemical nature of the surface (for example by adsorption), insufficient to cause any marked change in the weight of the particle.

Considering the tables and curves obtained in the investigation of the six proteins described above pp. 53—77 the following may be said about their uniformity as regards electrophoresis.

To ascertain if a protein is uniform or not, experiments at high mobilities are as important as those at low mobilities. The latter give information about the uniformity with respect to the isoelectric point. However, we may very well imagine that colloids exist consisting of a chemically uniform material with a well defined isoelectric point, the particles, however, being somewhat different as regards the frictional resistance in electrophoresis on account of differences, for example, in size or shape. This kind of non-uniformity should be revealed especially at high mobilities.

With regard to the isoelectric point uniformity, it may be seen from the tables that evidently well-defined isoelectric points were obtained, i.e. in no case migration towards both sides could be observed, as would have been the case if the proteins had been mixtures with a difference in  $p_H^\circ$  larger than the difference between the found  $p_H^\circ$  and the  $p_H$  at which the experiment was made. This conclusion is supported by a comparison of the photometric records obtained from the experiments in the isoelectric region with those obtained from mixtures of artificial proteins just described. In no one of the experiments described could any marked non-uniformity be revealed. This result was, however, not obtained for all proteins at once. Some examples have already been given, where the material investigated was found non-uniform, and to these many others may be added where uniform migration was first obtained since fresh material had been prepared or the old material first investigated had been recrystallized.

The boundary anomalies having been depressed, the diffusion is of course the main source of uncertainty when attempts are made to gain information about uniformity, especially since it has not been possible with the present technique to account quantitatively for this influence. Consequently one can only take such effects into account as are much larger than the diffusion. It is therefore a great advantage that the influence of the diffusion may be obtained separately by allowing the boundary to migrate back to the position at start, as described in several experiments in the preceding. Cases when the slope of the curves in the diagrams steadily decreased at both boundaries during the experiment, even if the current was reversed were therefore regarded to represent uniform migration within the experimental errors, whereas cases where the slope increased again, when the direction of the current was reversed, or any cases of reversible blurring or separation into several boundaries, were regarded as representing non-uniform migration, in analogy to the effects obtained with artificial mixtures.

It is evident that the question whether the proteins are really absolutely uniform or not cannot be answered on the basis of the experiments described, especially not in the case of the proteins with high diffusion constants: egg albumin, serum albumin, and Bence Jones protein. For the proteins with low diffusion constants: phycoerythrin, phycocyan, and hemocyanin the experiments must be considered to give evidence of a high degree of uniformity. See, for example, the experiment no. 3 with hemocyanin in acetate buffer p. 63 in a  $p_H$  distance of only 0.02 from the isoelectric point, the current being sent through for 441 minutes. This indicates a high degree of uniformity as regards the isoelectric point. Also fig. 32 referring to the experiment with the same substance at a high mobility in phosphate buffer shows uniform migration. In the same way the phycoerythrin experiments, p. 62 and p. 95, and the experiments with phycocyan evidently indicate a decidedly uniform migration. Egg albumin and Bence Jones protein did not at any rate give any clear evidence of non-uniformity. Serum albumin in the experiment no. 4, p. 59, showed a decided non-uniformity and also the other experiments with this protein showed signs of inhomogeneity.

### The electrophoresis of serum globulin.

It was originally intended to study the electrophoresis of serum globulin as well as that of the other proteins, but as it was found markedly non-uniform even after fractionation, further work was confined to some experiments to demonstrate this uniformity.

There has been a large amount of work and discussion on this subject, on account of the great physiological importance of the substance. The serum globulin has not hitherto been obtained in crystalline form, and, as has been especially

studied by SØRENSEN (96, 97, 98), it does not show a well defined solubility like egg albumin: the concentration of protein in solution depending upon the quantity of solid substance in contact with the solution. By precipitation with ammonium sulphate or by dialysis it can be obtained in different fractions showing different solubilities. The water-soluble fractions are generally called pseudoglobulin and the salt-soluble euglobulin. However, neither of these fractions shows a well-defined solubility. They differ not only in this property but also in phosphorus content (96) and light absorption (69).

To explain the anomalous behaviour of this protein SØRENSEN made the assumption that serum globulin is a mixture of at least two components which in solution as well as in solid form are partially bound by each other, therefore making a complete separation difficult or impossible.

In electrophoresis measurements like those discussed in the preceding section we have a direct means of studying the state of this substance in solution, from the point of view of uniformity.

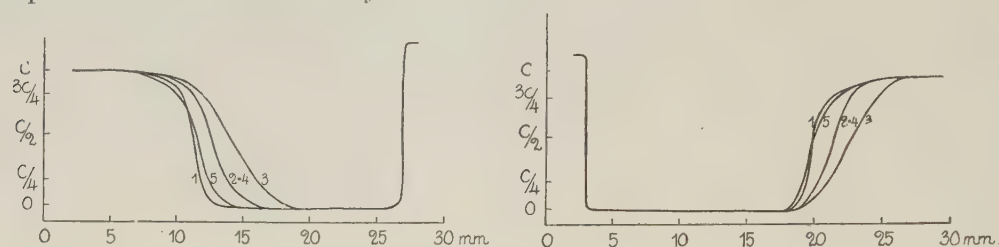


Fig. 45.

Fig. 45 and table LXXVII give the results of an experiment with (unfractionated) serum globulin (preparation see above p. 51).

Table LXXVII.

0.26 % serum globulin in phosphate buffer 51.13 cm.<sup>3</sup> 0.2 m Na<sub>2</sub>HPO<sub>4</sub> + 23.73 cm.<sup>3</sup> 0.2 m KH<sub>2</sub>PO<sub>4</sub>, diluted to 500 cm.<sup>3</sup>.  $p_H = 7.22$ .  $\alpha = 3.65 \times 10^{-3}$ .

Migration anodical.

| $i \times 10^3$ | $t$ | $\Delta_L$ | $\Delta_R$ | $\Delta_M$ | $u_{20} \times 10^5$ |
|-----------------|-----|------------|------------|------------|----------------------|
| 2.040           | 60  | +1.40      | -1.72      | 1.56       | 5.9                  |
| 2.041           | 60  | +1.22      | -1.24      | 1.23       | 4.7                  |
| 2.041           | 60  | -1.22      | +1.24      | 1.23       | 4.7                  |
| 2.053           | 60  | -1.24      | +1.46      | 1.35       | 5.1                  |
|                 |     |            |            |            | 5.1                  |

The mobility was calculated from the  $c/2$ -movement, even though in this case the meaning of the quantity obtained is very doubtful. The values also were rather irregular.



The substance shows decidedly inhomogeneous migration. The same result was obtained in an experiment in acetate buffer of  $p_H = 4.67$ . These experiments confirm the view that serum globulin in solution is not a chemical individual.

Fractions of pseudoglobulin and euglobulin were also investigated, in order to decide whether these proteins in solution are uniform or not. They were prepared by dialyzing the serum globulin stock solution for a week against distilled water at  $5^\circ$  and then electro dialyzing the solution for 36 hours with a current density of 0.5 milliamp./cm<sup>2</sup>. The clear filtrate of the solution after this treatment was the pseudoglobulin fraction. The precipitate from the dialysis and electro dialysis was dissolved in a phosphate buffer of  $p_H = 5.5$ , 0.2 m in total phosphate and dialyzed against the same buffer for a week. This preparation was carried out by B. SJÖGREN.

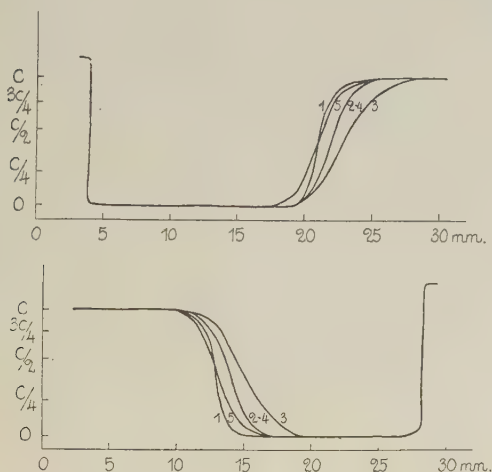


Fig. 46.

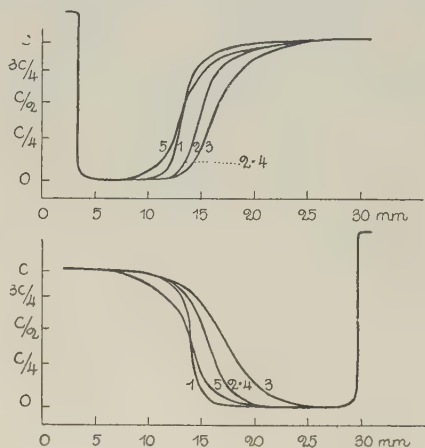


Fig. 47.

The results of the experiments with these fractions are given in fig. 46—47, and tables LXXVIII—LXXIX. The experiments were carried out in a buffer solution of the same composition as was used in the experiment with the unfractionated globulin.

Table LXXVIII.

0.2 % pseudoglobulin. Buffer equal to the solution given in table LXXVI.

Migration anodical.

| $i \times 10^3$ | $t$ | $\Delta_L$ | $\Delta_R$ | $\Delta_M$ | $u_{20} \times 10^5$ |
|-----------------|-----|------------|------------|------------|----------------------|
| 2.060           | 60  | +0.94      | -1.00      | 0.97       | 3.6                  |
| 2.053           | 60  | +0.74      | -1.14      | 0.94       | 3.5                  |
| 2.060           | 60  | -0.74      | +1.14      | 0.94       | 3.5                  |
| 2.060           | 60  | -0.90      | +0.82      | 0.86       | 3.2                  |
|                 |     |            |            |            | 3.5                  |

Table LXXIX.

Euglobulin, concentration not determined. Buffer equal to the solution given in table LXXVI. Migration anodical.

| $i \times 10^3$ | $t$ | $A_L$ | $A_R$ | $A_M$ | $u_{20} \times 10^5$ |
|-----------------|-----|-------|-------|-------|----------------------|
| 2.040           | 60  | +1.40 | -1.70 | 1.55  | 5.9                  |
| 2.040           | 60  | +1.38 | -1.70 | 1.54  | 5.8                  |
| 2.040           | 60  | -1.38 | +1.70 | 1.54  | 5.8                  |
| 2.040           | 60  | -1.50 | +1.70 | 1.60  | <u>6.1</u>           |
|                 |     |       |       |       | 5.9                  |

It is seen that also the fractions are non-uniform and in this respect not markedly different from the unfractionated serum globulin. The »mobilities» are, however, different from each other and from that of the unfractionated material, which has a mobility between those of the fractions.

With the same material and at the same time ultracentrifugal investigations were made by SJÖGREN (69). He found the unfractionated material uniform as regards molecular weight, whereas the fractions were both strongly non-uniform. The fractionation process was found to be irreversible: the sedimentation of a mixture of the two fractions was different from that of the original serum globulin. From these results it is evident that the fractionation process is not only a separation but also involves irreversible changes in the state of aggregation of the products.

The fact that unfractionated serum globulin is uniform in sedimentation but non-uniform in electrophoresis indicates an inhomogeneity of the kind discussed above (p. 96).

It should be mentioned in this connection that CHICK (99) put forward the view that the anomalous behaviour of serum globulin as regards solubility is caused by the presence of a serum lipoid, partially bound by the protein. It seems possible that such a change of the protein molecule might influence the electrophoresis more than the sedimentation.

### Summary.

1) The development of the moving boundary method for measurement of electrophoresis has been reviewed.

2) A method has been described for measurement of the electrophoretic mobility of proteins, especially at low mobilities in the neighbourhood of the isoelectric points. The method mainly consists in photographing with ultraviolet light the boundary movement in a quartz U-tube apparatus. The photographic images are registered in a microphotometer in enlarged scale, and the movement of the boundaries obtained from measurements of the displacement of the photometric record curves.

3) The sources of error of the moving boundary method in general have been discussed. Special attention has been devoted to the effects causing deformation of the boundaries («the boundary anomalies»). By the aid of the method just mentioned it has been possible to demonstrate the influence of the sources of errors experimentally.

4) Six comparatively well defined proteins have been studied. The preparation of these proteins has been described.

5) The results of the measurements of the mobilities in the isoelectric regions are given in tables and diagrams.

6) From the diagrams the isoelectric points may be obtained with comparatively great accuracy. It has been found that the position of the isoelectric point is to some extent dependent upon the medium. It has further been found that at least for egg albumin there is a marked discrepancy between the isoelectric point and the point of minimum acid-base combining capacity. A possible explanation of this result is discussed.

From the mobilities in the isoelectric region and the molar frictional coefficients determined with the ultracentrifuge the apparent charge of the proteins has been calculated. The relation of this charge to the actual charge has been discussed from the standpoint of the DEBYE-HÜCKEL theory of electrophoresis.

7) The possibility of using moving boundary electrophoresis as a criterion of chemical uniformity of proteins has been demonstrated with experiments on artificial mixtures. Examples have been given of cases when it has been possible to show with this method that proteins are non-uniform or impure. It has been found that

proteins may be uniform as regards particle size but non-uniform as regards mobility and vice versa. Serum globulin has been found markedly non-uniform in electrophoresis, even after fractionation.

This investigation was carried out in the Laboratory of Physical Chemistry, Upsala. To the Chief of the Laboratory, Professor THE SVEDBERG, the author wishes to express his deep gratitude for suggesting this research and for his kind interest in its performance.

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